



# LUND UNIVERSITY

## On the role of mast cells in chemokine-induced leukocyte recruitment

Wang, Yusheng

2006

[Link to publication](#)

*Citation for published version (APA):*

Wang, Y. (2006). *On the role of mast cells in chemokine-induced leukocyte recruitment*. [Doctoral Thesis (compilation), Surgery]. Department of Clinical Sciences, Lund University.

*Total number of authors:*

1

### General rights

Unless other specific re-use rights are stated the following general rights apply:

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

Read more about Creative commons licenses: <https://creativecommons.org/licenses/>

### Take down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

LUND UNIVERSITY

PO Box 117  
221 00 Lund  
+46 46-222 00 00

# **On the Role of Mast Cells in Chemokine-Induced Leukocyte Recruitment**

Yusheng Wang

Academic Thesis

With permission from the Medical Faculty at Lund University for the presentation of this PhD thesis in a public forum in the CRC, Entrance 72, Malmö University Hospital, Malmö, on Thursday, 26<sup>th</sup> October 2006 at 1 pm.

Faculty opponent: Johan D Söderholm, MD, PhD, Associate Professor,  
Linköping University

Supervisor: Henrik Thorlacius, MD, PhD, Associate Professor



**LUND UNIVERSITY**  
Faculty of Medicine

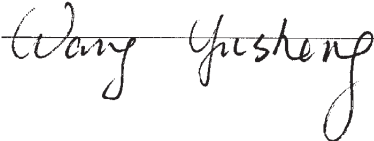
Malmö 2006

Department of Clinical Sciences, Surgery Research Unit,  
Malmö University Hospital

Organization LUND UNIVERSITY  Department of Surgery Malmö University Hospital SE-205 02 Malmö		Document name DOCTORAL DISSERTATION
		Date of issue Oct 26th 2006
Author(s) Yusheng Wang		Sponsoring organization
Title and subtitle On the Role of Mast Cells in Chemokine-Induced Leukocyte Recruitment		
Abstract Leukocyte recruitment is considered to play a key role in numerous inflammatory diseases. Tissue accumulation of leukocytes is a multi-steps process comprising rolling, adhesion and transmigration. This process depends on the function of several different cells, including leukocytes, endothelial cells, mast cells and macrophages as well as a wide spectrum of inflammatory mediators, such as TNF-alpha and chemokines. Chemokines are effective leukocyte attractants and according to structural properties, chemokines are divided into two major subfamilies, CC and CXC. In general, CC chemokines exert chemotactic influence on monocytes and lymphocytes while CXC chemokine mainly attract neutrophils. However, the mechanisms of chemokine-induced leukocyte recruitment in vivo remain elusive. Mast cells are tissue resident cells exerting multi-functional roles. Upon activation, mast cells release potent mediators, such as histamine, leukotrienes, chemokines and TNF-alpha. The aim of this study was to investigate the role of mast cells in regulating chemokine-provoked tissue infiltration of leukocytes. It was found that TNF-alpha can activate endothelial cells to express P-selectin and CXC chemokine (MIP-2) and cause leukocyte adhesion in vitro in a glucocorticoid-sensitive manner. Interestingly, chemokines, both MIP-1alpha, MCP-1 and MIP-2, KC, were found to provoke a dose-dependent increase in P-selectin-dependent leukocyte rolling, adhesion and recruitment in vivo. It was demonstrated that chemokine-induced neutrophil recruitment in vivo was abolished in mast cell-deficient mice. TNF-alpha activated endothelial cells (increased E-selectin mRNA expression) in both wild-type and mast cell-deficient animals. In contrast, MIP-1alpha, MCP-1 and MIP-2 increased gene expression of E-selectin only in wild-type mice but not in mast cell-deficient mice, suggesting that chemokine-induced activation of endothelial cells is indeed dependent on mast cells. Moreover, it was observed that mast cell-derived TNF-alpha, but not histamine and leukotrienes, mediated chemokine-induced extravasation of neutrophils. Considered together, this work elucidates important mechanisms regulating chemokine-induced leukocyte recruitment, which may help understand the pathophysiology of inflammatory diseases and serve as a basis for development of anti-inflammatory treatment strategies.		
Key words: neutrophil, recruitment, adhesion, chemokines, mast cells, TNF-alpha, dexamethasone, P-selectin, E-selectin, activation, endothelial cells		
Classification system and/or index terms (if any): Lund University, Faculty of Medicine Doctoral Dissertation Series 2006:113		
Supplementary bibliographical information:		Language English
ISSN and key title: 1652-8220		ISBN 91-85559-37-7
Recipient's notes	Number of pages	Price
	Security classification	

Distribution by (name and address)

I, the undersigned, being the copyright owner of the abstract of the above-mentioned dissertation, hereby grant to all reference sources permission to publish and disseminate the abstract of the above-mentioned dissertation.

Signature 

Date 29th Aug. 2006

To: Qing

ISSN 1652-8220

ISBN 91-85559-37-7

Lund University, Faculty of Medicine Doctoral Dissertation Series 2006:113

Printed by Media-Tryck, Lund University

Lund, 2006



## CONTENTS

1. LIST OF ORIGINAL PAPERS	3
2. ABBREVIATIONS	4
3. INTRODUCTION	5
4. GENERAL BACKGROUND	6
<b>4.1. Leukocyte recruitment</b>	6
4.1.1. Leukocyte recruitment is a multistep process	6
4.1.2. Leukocyte rolling is a precondition in leukocyte recruitment	7
4.1.3. Endothelial cells and activation	7
<b>4.2. Cell adhesion molecules</b>	9
4.2.1. Selectins and leukocyte rolling	9
4.2.2. Integrins and ICAM-1 in firm adhesion	12
4.2.3. PECAM-1 and transmigration	13
<b>4.3. Pro-inflammatory mediators</b>	14
4.3.1. TNF- $\alpha$	14
4.3.2. Chemokines	15
<b>4.4. Mast cells</b>	18
4.4.1. Role of mast cells in disease	18
4.4.2. Distribution and subtypes of mast cells	19
4.4.3. Activation of mast cells	19
4.4.4. Mediators of mast cells	21
<b>4.5. Glucocorticoids</b>	22
5. AIMS OF THE THESIS	24

6. MATERIALS AND METHODS	25
6.1. <b>Animals</b>	25
6.2. <b>Cells</b>	25
6.2.1. Bone marrow mast cells (BMMC)	25
6.2.2. Neutrophil isolation	26
6.2.3. Endothelial cells	26
6.3. <b>Intravital microscopy</b>	26
6.4. <b>Histology</b>	28
6.5. <b>Cell adhesion assay</b>	28
6.6. <b>Flow cytometry</b>	29
6.7. <b>RT-PCR</b>	29
6.8. <b>ELISA</b>	30
6.9. <b>Statistic analysis</b>	31
7. RESULTS AND DISCUSSION	32
8. SUMMARY (SWEDISH)	43
9. CONCLUSIONS	45
10. ACKNOWLEDGMENTS	46
11. REFERENCES	47
12. PAPERS	59



## 1. LIST OF ORIGINAL PAPERS

This thesis is based on the following papers:

I. Qing Liu, **Yusheng Wang**, Henrik Thorlacius.

Dexamethasone inhibits tumor necrosis factor- $\alpha$ -induced expression of macrophage inflammatory protein-2 and adhesion of neutrophils to endothelial cells.

*Biochemical and Biophysical Research Communications* (2000) **271**: 364-367

II. Xiaowei Zhang, Qing Liu, **Yusheng Wang**, Henrik Thorlacius.

CXC chemokines, MIP-2 and KC, induce P-selectin-dependent neutrophil rolling and extravascular migration *in vivo*.

*British Journal of Pharmacology* (2001) **133**: 413-421

III. Ming Xiu Wan\*, **Yusheng Wang\***, Qing Liu, Rene Schramm, Henrik Thorlacius.

CC chemokines induce P-selectin-dependent neutrophil rolling and recruitment *in vivo*: intermediary role of mast cells.

*British Journal of Pharmacology* (2003) **138**: 698-706

\*These authors contributed equally to this work

IV. **Yusheng Wang**, Henrik Thorlacius.

Mast cell-derived tumor necrosis factor- $\alpha$  mediates macrophage inflammatory protein-2-induced recruitment of neutrophils in mice.

*British Journal of Pharmacology* (2005) **145**: 1062-1068

## 2. ABBREVIATIONS

BMMC	bone marrow mast cell
CD	cluster of differentiation
CMP48/80	compound 48/80
ELC	EBI1-ligand chemokine
FITC	fluorescein isothiocyanate-conjugated
fMLP	N-formyl-methionyl-leucyl-phenylalanine
GRO- $\alpha$	growth-related oncogene- $\alpha$
HCC	hemofitrate CC chemokine
ICAM	intercellular adhesion molecule
IL	interleukin
i.p.	intraperitoneal
IP-10	IFN- $\gamma$ -inducible protein-10
KC	cytokine-induced neutrophil chemoattractant
MCP-1	monocyte chemotactic protein-1
MDC	macrophage-derived chemokine
MIG	monokine induced by IFN- $\gamma$
MIP-1 $\alpha$	macrophage inflammatory protein-1 $\alpha$
MIP-2	macrophage inflammatory protein-2
NAP-2	neutrophil-activating protein-2
PBS	phosphate buffered saline
PECAM-1	platelet endothelial cell adhesion molecule-1
PMNL	polymorphonuclear leukocyte
RANTES	regulated upon activation, normal T cell expressed and secreted
RT-PCR	reverse-transcription polymerase chain reaction
SDF-1	stromal-derived factor-1
TARC	thymus and activation-regulated chemokine
TNF- $\alpha$	tumor necrosis factor- $\alpha$

### 3. INTRODUCTION

Inflammation is a tissue reaction to microbial invasion or non-self substances and vital to the survival of all complex organisms but plays also a key role in numerous inflammatory diseases. The inflammatory reaction is characterized by classical signs of pain (*dolor*), heat (*calor*), redness (*rubor*), swelling (*tumor*), and loss of function (*functio laesa*). All these clinical signs of inflammation are reflections of a series of overlapping changes in the tissue microcirculation, including vasodilatation, plasma leakage and leukocyte recruitment into the inflammatory focus. Indeed, leukocyte recruitment is a central event in the pathogenesis of most types of inflammatory disease. Diseases characterized by exaggerated inflammation are an increasingly important cause of morbidity and mortality in human medicine. A more sophisticated understanding and capability to control these complex inflammatory mechanisms may offer an opportunity to improve the treatment of patients suffering from pathological inflammation. Mast cells are tissue resident cells capable of secreting numerous powerful mediators and their close proximity to external surfaces, such as the skin and mucosal epithelium in the respiratory and gastrointestinal tract as well as the tissue blood vessels make them suitable for regulating the inflammatory process and of key interest in pathophysiological research. However, the detailed role of mast cells in regulating complex interactions between tissue cells and leukocytes in the blood remain elusive. The purpose of this work was to better define the role of mast cells in initiating and mediating leukocyte recruitment *in vivo*.

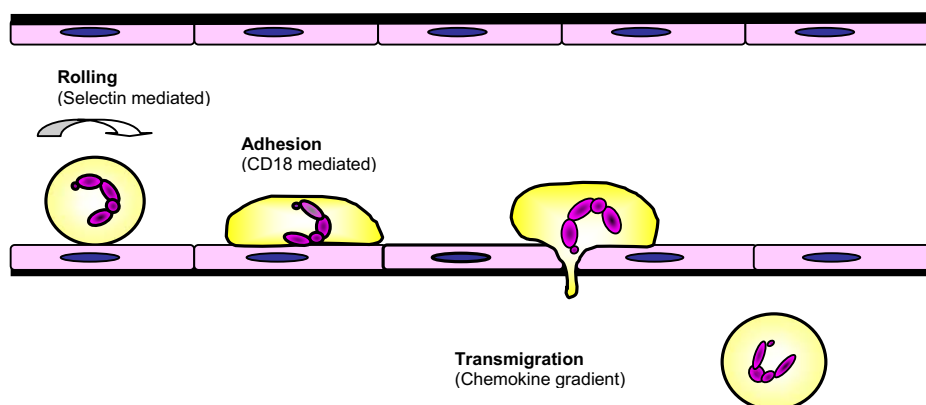
## 4. GENERAL BACKGROUND

### 4.1. Leukocyte recruitment

#### 4.1.1 Leukocyte recruitment is a multistep process

Tissue infiltration of leukocytes from the circulating blood into the extravascular space is a key feature of the inflammatory response. Leukocyte recruitment is mainly composed of leukocyte tethering, rolling, adhesion and transmigration (Blankenberg *et al* 2003). These leukocyte-endothelium interactions are supported by specific cell adhesion molecules expressed on different cells. Initially, leukocyte rolling is enabled by relatively weak and reversible adhesions and mediated by the selectin family of cell adhesion molecules. Firm adhesion of leukocytes to endothelial cells is mainly mediated by  $\beta$ 2-integrins (CD18) binding to members of the immunoglobulin supergene family. The last step named transmigration is largely supported by PECAM-1 (CD31) (Ebnet *et al* 1999).

Figure 1. Leukocyte recruitment is composed of the leukocyte rolling, adhesion and transmigration



#### 4.1.2 Leukocyte rolling is a precondition in leukocyte recruitment

Leukocyte rolling is the initial step and has been demonstrated to be a precondition for the subsequent firm adhesion and transmigration (Lindbom *et al* 1992, Mansson *et al* 2000). This rolling interaction is mediated by the selectin family of adhesion molecules. One investigation showed that inhibition of the selectins abolished not only leukocyte rolling but also firm adhesion and extravascular recruitment (Lindbom *et al* 1992). Another study reported that leukocyte adhesion and transmigration was reduced when leukocyte rolling was inhibited by enzymatic removal of L-selectin (von Andrian *et al* 1992). Notably, a key study demonstrated that leukocyte rolling abolished by an anti-P-selectin antibody can completely inhibit leukocyte adhesion and recruitment in TNF- $\alpha$ -activated tissue (Mansson *et al* 2000). One important aspect of leukocyte rolling is the velocity reduction, which facilitates detection of chemoattractant molecules either exposed on the endothelial cell lining or leaking between adjacent endothelial cells from the inflamed tissue (Jung *et al* 1999). Chemokines are **chemo**attractant **cytokines**, which directly activate a rolling leukocyte and convert it to a firmly adherent one. Although circulating leukocytes are main targets of most chemokines, an increasing literature has shown that also other cells, such as tissue resident macrophages and mast cells, express chemokine receptors and may mediate indirect effect of relevance to leukocyte recruitment *in vivo*. Nonetheless, it is pivotal to this work to note that leukocyte rolling is a prerequisite for leukocyte recruitment and that leukocyte rolling is dependent on endothelial cell activation and selectin expression.

#### 4.1.3 Endothelial cells and activation

The vascular endothelium is a single-cell layer that forms a continuous lining for the large container that holds the circulating blood. One main function of endothelial cells is to provide a homeostatic barrier between blood and tissues preventing thrombosis formation. However, as outlined herein, endothelial cell

activation plays a pivotal role in initiating and supporting leukocyte recruitment. Various agonists, including physical factors, invading microbes, complement factors and cytokines, such as TNF- $\alpha$  may activate endothelial cells. In leukocyte recruitment, the concept of endothelial cell activation must be considered with the following event, in which endothelial cells begin to express P- and E-selectin in order to initiate leukocyte rolling. Since leukocyte rolling is a critical precondition in leukocyte recruitment (Lindbom *et al* 1992, von Andrian *et al* 1992; Mansson *et al* 2000), it is obvious that endothelial cell activation is also of critical importance in the leukocyte recruitment process, *i.e.* without endothelial cell activation, no expression of selectins on endothelial cells and no rolling adhesive interaction. Thus, activation of endothelial cells exerts a key role in leukocyte recruitment by supporting leukocyte rolling. TNF- $\alpha$  is a powerful inducer of leukocyte recruitment and has the capacity to induce all steps in the extravasation process of leukocytes, including leukocyte rolling (endothelial cell activation including P-selectin expression) (Mansson *et al* 2000). As we know, activation and trafficking of leukocytes are regulated by chemokines (Zlotnik *et al* 1999). However, it is not known whether chemokines can induce all steps in the extravasation process of leukocytes *in vivo*. In particular, it is unknown whether chemokines can induce leukocyte rolling, which would require endothelial cell activation as mentioned above. The literature on the expression of chemokine receptors on endothelial cells is complex and contradictory. Several authors reported that endothelial cells do not express chemokine receptors and, thus chemokines should be unable to activate endothelial cells directly *in vitro* (Schonbeck *et al* 1995, Petzelbauer *et al* 1995; Gupta *et al* 1998). But another author indicated endothelial cells do express CXC chemokines receptors mRNA but the endothelial cells didn't respond to CXC chemokine, such as IL-8 and GRO- $\alpha$  (Murdoch *et al* 1999). As the activation of endothelial cells is a prerequisite in leukocytes rolling, it is necessary to know if chemokines can

directly activate endothelial cells or not and how chemokines may contribute to leukocyte-endothelium reactions *in vivo*.

Considering this, one would not expect endothelial cells to express chemokine receptors, for example CXCR2, in the case of MIP-2- and KC-induced leukocyte recruitment. Alternatively, CXC chemokines may stimulate an intermediate tissue cell that both express CXCR2 and is capable of secreting pro-inflammatory mediators with the capacity to stimulate endothelial cell activation and P-selectin expression in order to initiate and support leukocyte rolling. Herein, we hypothesized that mast cells may fulfill these criteria in the inflammatory process.

#### **4.2 Cell adhesion molecules**

According to the definition of the Gene Ontology Consortium, cell adhesion molecules are molecules expressed on the surface of a cell that mediate adhesion of the cell to other cells or to the extracellular matrix (Blankenberg *et al* 2003). These proteins are playing numerous crucial functions at the interface of a cell and its environment, whether this environment is another cell, from a similar or different origin or extracellular matrix or even sometimes soluble elements. Adhesion molecules are widely distributed and virtual every cell expresses cell adhesion molecules. Some adhesion molecules are the biochemical fundament in leukocyte recruitment and are listed in Table 1.

##### **4.2.1 The selectins and leukocyte rolling**

The selectin family of adhesion molecules contains three members, L-, P- and E-selectin. L- refers to leukocyte; P- to platelet and E- to endothelial cell (Carios *et al* 1994, Vestweber *et al* 1999). All selectins have five common structures: (1) the selectin-like function of NH<sub>2</sub>-terminal domain; (2) an epidermal growth factor (EGF)-like part; (3) 2-9 fragments of short consensus repeat; (4) a transmembrane domain; (5) a cytoplasmic tail (Lasky 1995, Bevilacqua *et al* 1994, McEver 1994). Each selectin may recognize and bind to a very restricted number of high-affinity

ligands individually *in vivo* (see Table 1.). P-selectin glycoprotein ligand (PSGL)-1 has been shown to be the dominant physiologic ligand for P-selectin. In fact, all

Table 1. The main cell adhesion molecules involved in leukocyte recruitment

<b>Adhesion molecules</b>	<b>Ligands</b>	<b>Functions</b>	<b>Distribution</b>
<b><i>Selectins</i></b>			
L-selectin	GlyCAM-1; CD34	Rolling	Leukocyte
E-selectin	ESL-1; PSGL-1	Rolling	Endothelial cell
P-selectin	PSGL-1	Rolling	Endothelial cell
<b><i>Selectin-ligands</i></b>			
ESL-1	E-selectin	Rolling	Leukocyte
PSGL-1	P-selectin	Rolling	Leukocyte
<b><i>Immunoglobulin superfamily</i></b>			
ICAM-1	$\alpha$ L $\beta$ 2; $\alpha$ M $\beta$ 2	Firm adhesion	Endothelial cell
ICAM-2	$\alpha$ L $\beta$ 2 $\alpha$ M $\beta$ 2	Firm adhesion	Endothelial cell
ICAM-3	$\alpha$ D $\beta$ 2	Firm adhesion	Endothelial cell
PECAM-1	PECAM-1	Transmigration	Endothelial cell Leukocyte
<b><i>Integrins</i></b>			
$\alpha$ L $\beta$ 2 (CD11a/CD18)	ICAM-1; ICAM-2	Firm adhesion	Leukocyte
$\alpha$ M $\beta$ 2 (CD11b/CD18)	ICAM-1; ICAM-2	Firm adhesion	Leukocyte
$\alpha$ D $\beta$ 2 (CD11c/CD18)	ICAM-3	Firm adhesion	Leukocyte

P-selectin-dependent leukocyte rolling *in vivo* is mediated by PSGL-1. PSGL-1 can also bind to E-selectin but with much lower affinity than to P-selectin and the physiologic role of PSGL-1/E-selectin interactions remains to be demonstrated. A specific E-selectin ligand (ESL-1) has also been identified but its function is not yet well known (Blankenberg *et al* 2003).



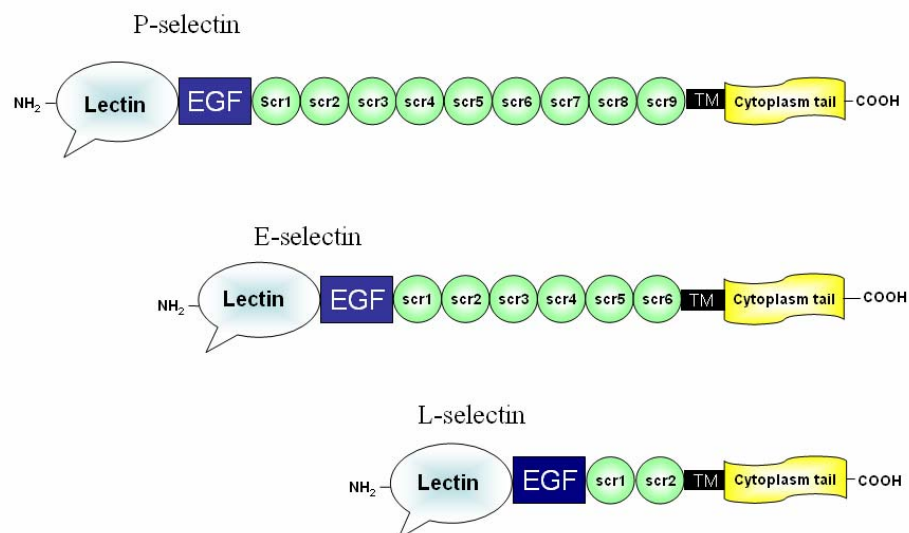
The molecular weight of L-selectin is dependent on the level of glycosylation from 74K (lymphocyte) to 110K (neutrophil). It is only expressed in hematopoietic cells as a constitutive component of the surface of the leukocyte. L-selectin may mediate not only binding to an inducible vascular ligand(s) but also to yet undefined ligands at sites of inflammation but mainly is recognized for its important role in the recirculation of lymphocytes through peripheral lymphoid tissues (Baggiolini *et al* 1999).

P-selectin is a 140K protein and stored in Weibel-Palade bodies in endothelial cells and in  $\alpha$ -granules of platelets (Hsu-Lin *et al* 1984, McEver *et al* 1989). Following activation by thrombogenic or inflammatory mediators,  $\alpha$ -granules or Weibel-Palade bodies containing P-selectin fused with the plasma membrane, thereby rapidly exposing P-selectin on the cell surface within 2 min. P-selectin expression is generally short-lived reaching its peak after only 5-10 min, declining to baseline after 3h, which is consistent with its role in mediating early leukocyte-endothelial cell interactions. However, besides the preformed P-selectin stored in Weibel-Palade bodies, additional synthesis and expression of P-selectin at the gene level may be mobilized within 2h by cytokine stimulation (IL-1, TNF- $\alpha$ ) (Blann *et al* 2004). Indeed, *in vivo* studies of P-selectin function suggest that it may also be important at later time points as a cytokine-induced adhesion molecule (Weller *et al* 1992, Robinson *et al* 1999, Kanwar *et al* 1999, Mansson. 2000).

The molecular weight of E-selectin is nearly 115K. Only endothelial cells can express E-selectin when stimulated with TNF- $\alpha$ , IL-1 or lipopolysaccharide (LPS) (Bevilacqua *et al* 1987, 1989). In resting endothelial cells, there is no E-selectin expression. Generally, it takes one hour for initial expression of E-selectin in endothelial cells and after 4-6 hours maximum expression is reached. E-selectin expression may be sustained for 24-48 hours. It has been suggested that E-selectin plays a redundant role together with P-selectin in cytokine-activated tissues (Tang *et al* 1996), however, more recent data suggest that P-selectin is the dominant

rolling receptor in inflamed venules and that E-selectin may be more important for events downstream of leukocyte rolling, such as an intermediate signaling function in between rolling and firm adhesion. Nonetheless, expression of E-selectin may also be used as a marker of endothelial cell activation.

Figure. 2. Selectin structure. The lectin domain, epidermal growth factor (EGF)-like domain, short consensus repeat (SCR) fragment, Transmembrane (TM) domain, and cytoplasm tail



#### 4.2.2 Integrins and ICAM-1 in leukocyte firm adhesion

When rolling leukocytes are activated by chemokines, integrins are activated and upregulated, resulting in firm adhesion between the leukocyte and endothelial cells. Integrins are heterodimeric transmembrane glycoproteins formed by the non-covalent association of one  $\alpha$ -subunit and one  $\beta$ -subunit. The integrins are

expressed in a large variety of cells where they regulate many diverse functions, including growth, differentiation, motility and polarity (Kishimoto *et al* 1999) but only integrins relevant to the present work on leukocyte recruitment will be mentioned here. Integrins have exerted various states of activation, *i.e.* in resting cells they are usually in a non-adhesive conformation that provides low affinity for ligands. Changes in cell activity may trigger signals that modify their conformation to develop a high affinity. Activated integrins mediate firm adhesion by recognizing members of the immunoglobulin gene superfamily expressed on the endothelial cell surface, such as ICAM-1, ICAM-2 and VCAM-1 (Table 1).  $\beta 2$  (CD18) or “leukocyte” integrins are restricted to leukocytes and as adhesion receptors they are fundamental to both innate and adaptive immunity. Thus, the integrins involved in leukocyte firm adhesion to endothelial cells are mainly of the  $\beta 2$ -integrin (CD18) subfamily, such as  $\alpha L\beta 2$  (LFA-1 or CD11a/CD18);  $\alpha M\beta 2$  (Mac-1 or CD11b/CD18);  $\alpha D\beta 2$  (p150, 95 or CD11c/CD18). Moreover,  $\alpha 4\beta 1$  (VLA-4) expressed on lymphocytes and activated neutrophils (Blankenberg 2003) appear to play a role in the recruitment process by interacting with VCAM-1 on endothelial cells. Intercellular adhesion molecules (ICAMs) belong to the immunoglobulin (Ig) superfamily, which are membrane glycoprotein receptors containing a variable number of extracellular Ig-like domains. ICAM-1 is widely expressed at a basal level and can be up-regulated by pro-inflammatory cytokines on endothelial cells while ICAM-2 is present in high levels in resting endothelial cell and not inducible.

#### 4.2.3 PECAM-1 and transmigration

Transendothelial migration of leukocytes is the last step of recruitment process of leukocytes by which the leukocyte squeezes in an amoeboid fashion across the endothelial cell barrier and moves to the site of maximal inflammation guided by concentration gradients of chemoattractant molecules. This transendothelial passage is believed to occur along the junctions between tightly apposed

endothelial cells. Some authors believe that there is a transcellular pathway through which leukocytes may directly migrate through endothelial cells (Engelhardt *et al* 2004). Platelet endothelial cell adhesion molecule-1 (PECAM-1; CD31) belongs also to the Ig-like subfamily and exerts a unique role in the transmigration of leukocytes. It is expressed abundantly by endothelial cells and largely concentrated to the junctions between adjacent endothelial cells. PECAM-1 is also distributed on the surface of most leukocytes as well as platelets. Blockade of PECAM-1 interferes with the infiltration of leukocytes without affecting adhesion of leukocytes to endothelial surface. During leukocyte transmigration, PECAM-1 on leukocytes and endothelial cells interacts in a homophilic manner (Muller *et al* 1999; 2003).

### 4.3 Pro-inflammatory mediators

#### 4.3.1. TNF- $\alpha$

TNF- $\alpha$  is a powerful regulator of acute inflammation and leukocyte recruitment in numerous diseases. It is mainly produced by macrophages activated by LPS *in vivo*. But also epithelial cells and mast cells have ability to secrete TNF- $\alpha$  as well. TNF- $\alpha$  has two active forms including a secreted and membrane-bound form. TNF- $\alpha$  receptors are present on virtually all cell types of body implicating that TNF- $\alpha$  exerts widely different biological actions although it is mainly recognized for its potent pro-inflammatory and pro-apoptotic effects. Another well-known function is that TNF- $\alpha$  can induce necrosis in certain tumor cells for which it was once named tumor necrosis factor. In the context of leukocyte recruitment, one of the key effects of TNF- $\alpha$  major effect is to activate cells to express adhesion molecules and chemokines. Thus, TNF- $\alpha$  alone has the ability to induce the whole leukocyte recruitment process, including leukocyte rolling, adhesion and transmigration *in vivo* (Smart *et al* 1994, Tessier *et al* 1997, McColl *et al* 1999). Indeed, TNF- $\alpha$  plays a fundamental role in multiple inflammatory diseases including rheumatoid arthritis, Crohn's disease, autoimmune disorders and

surgical injury (van Deventer 1997, Klinkert *et al* 1997, Ksontini *et al* 1998, Moore *et al* 2000). It has been shown that TNF- $\alpha$  can promote leukocyte-endothelial cell interactions by increasing expression of adhesion molecules, including P- and E-selectin as well as ICAM-1 and VCAM-1 on endothelial cells (Bevilacqua 1987, Ley *et al* 1995, Pober *et al* 1986, Haraldsen *et al* 1996).

#### 4.3.2 Chemokines

Chemokines are a kind of cytokines stimulating leukocyte chemotaxis, *i.e.* **chemo**attractant **cytokines**. Chemokines are mainly involved in leukocyte recruitment although it is clear from a growing body of literature that chemokines are also important players in processes, such as angiogenesis, growth and adaptive immune functions (Rollins 1997). Tissue resident macrophages, mast cells, and recruited leukocytes as well as fibroblasts, endothelial cells or epithelial cells of the site of inflammation or infection can express and release large amounts of chemokines. Potent stimulators of chemokine formation include mainly pro-inflammatory cytokines, such as IL-1, TNF- $\alpha$ , interferon- $\gamma$  (IFN- $\gamma$ ) and hematopoietic growth factors as well as bacterial toxins, including LPS (Baggiolini 1999). From a structural perspective, chemokines constitute a family of small peptides (8-12K) and according to their primary structure (amino acid residual sequence) and number of amino acid residuals between the last two N-terminal cysteines, chemokines are divided into two large subfamilies, *i.e.* CC and CXC chemokines. CC and CXC chemokines can attract different kinds of leukocytes individually. *In vitro*, CC chemokine mainly attracts monocytes, lymphocytes but not neutrophils (Bonecchi *et al* 1999, McColl *et al* 1993, Cheng *et al* 2001). The CXC chemokines are also subdivided according to the presence of the ELR motif (glutamic acid-leucine-arginine) preceding CXC in the N-terminus. The ELR-CXC chemokines predominantly act on the neutrophil while non-ELR-CXC chemokines are more specific for lymphocytes. The classic CXC chemokine is IL-8 which is secreted by various cells and acts as a main attractant

of neutrophils. In mice, MIP-2 (macrophage inflammatory protein-2) and KC (cytokine-induced neutrophil chemoattractant) are produced by macrophages activated by LPS and perform chemotactic functions on neutrophils similar to IL-8. In lack of an identical match to IL-8 in mice as yet, both MIP-2 and KC are defined as homologues to human IL-8 (Huang *et al* 1992, Tekamp-Olson *et al* 1990). Chemokine receptors are seven-transmembrane-domain proteins that signal through GTP-binding proteins (Baggiolini *et al* 1999). There are mainly two CXC chemokine receptors expressed in neutrophils, those are CXCR1 and CXCR2. CXCR1 can bind IL-8 but has low affinity to other ELR-CXC chemokines. CXCR2 binds mainly to ELR-CXC chemokines. So, in mice, the function of CXC chemokines, such as MIP-2 and KC are mediated via binding to CXCR2 (Huber *et al* 1991, Cacalano *et al* 1994, Jones *et al* 1997). Other CXC receptors such as CXCR3 are only expressed on activated T lymphocytes while CXCR4 appear widely on most hematopoietic cells and even some tissue cells (Baggiolini *et al* 1999). CC chemokine receptors are predominately expressed on monocytes and lymphocytes (Baggiolini *et al* 1999). It has been reported that mast cells can express different kinds of chemokine receptors (Table 2.) (Schramm and Thorlacius 2004, Juremalm *et al* 2005).

Up to date, CXC chemokine-dependent leukocyte infiltration has been implicated as a fundamental part of the pathogenesis of several important clinical conditions, such as endotoxemia-induced lung injury (Schmal *et al* 1996), glomerulonephritis (Feng *et al* 1995) and bacteria meningitis (Diab *et al* 1999). However, the detailed mechanism of action of CXC chemokine-induced leukocyte-endothelial cell interactions remains elusive. To understand the molecular details of CXC chemokine-provoked tissue infiltration of leukocytes *in vivo* would not only deepen our understanding of the pathophysiology but may also provide mechanistic information that may form a basis for development of new and more

Table 2. Chemokine receptors expressed in mast cells

Receptors	Ligands
CCR1	MIP-1 $\alpha$ , RANTES, MCP-2, HCC-1, HCC-2
CCR2	MCP-1, MCP-2, MCP-3, MCP-4, MCP-5
CCR3	MIP-1 $\alpha$ , RANTES, MCP-2, MCP-3; Eotaxin, HCC-1, HCC-2
CCR4	RANTES, TARC, MDC
CCR5	MIP-1 $\alpha$ , MIP-1 $\beta$ , RANTES
CCR6	MIP-3 $\alpha$
CCR7	ELC, SLC
CXCR1	IL-8
CXCR2	GRO- $\alpha$ , NAP-2, IL-8
CXCR3	MIG, IP-10, I-TAC
CXCR4	SDF-1
CX3CR	Fraktalkine

effective therapies directed against inflammatory diseases. On the other hand, CC chemokines, such as MIP-1 $\alpha$  (macrophage inflammatory protein-1 $\alpha$ ) and MCP-1 (monocyte chemoattractant protein-1) predominantly stimulate chemotaxis of lymphocytes and monocytes but not neutrophils. However, the majority of these studies on CC chemokines have been performed *in vitro* systems comprising only endothelial cells and subpopulations of leukocytes (Bonecchi *et al* 1999, McColl *et al* 1993, Cheng *et al* 2001). In fact, there is growing body of data supporting that CC chemokines may also be involved in neutrophil trafficking *in vivo* as well (Appelberg 1992, Shanley *et al* 1995, Matsukawa *et al* 1999, Diab *et al* 1999). It has been reported that treatment with antibodies to MIP-1 $\alpha$  and MCP-1 may reduce extravascular neutrophil recruitment in pathological inflammatory models

(Shanley *et al* 1995, Matsukawa *et al* 1999). Moreover, MIP-1 $\alpha$  deficient mice exhibit clear-cut defects in neutrophil responses in complex inflammatory reactions and experiment in CCR1 gene-targeted mice implicate a role of CCR1 in neutrophil trafficking (Domachowske *et al* 2000). Nonetheless, it is not known whether CC chemokines can activate endothelial cells *in vivo* and thereby support selectin expression and leukocyte rolling, which is a precondition for subsequent leukocyte recruitment (Lindbom *et al* 1992, von Andrian *et al* 1992, Mansson *et al* 2000).

#### 4.4 Mast cells

##### 4.4.1 Role of mast cells in disease

Mast cells are multifunctional and tissue residual cells. Mast cells are predominantly localized along the tissue microvasculature and have a unique capacity to coordinate several aspects of acute inflammation, such as vascular leakage, expression of adhesion molecules and leukocyte infiltration (Schramm and Thorlacius 2004), which is classically manifested as anaphylaxis, urticaria, angioedema, and acute exacerbations of asthma. The contribution of mast cells in allergic disease and many chronic inflammations, such as rheumatoid arthritis, multiple sclerosis is well known. Moreover, it has been reported that mast cell-deficient mice were more susceptible to experimentally induced lung bacterial infections than wild-type mice and provided evidence that mast cell-derived TNF- $\alpha$  is an important cytokine in the clearance of bacteria by inducing the influx of neutrophils into the infected sites (Malaviya *et al* 1996). Thus, mast cells may be of key value in innate immune responses to bacteria and ischemia/reperfusion as well as in adaptive immunity to parasites. On top of that, mast cells have been implicated in wound healing, collagen turnover and fibrogenesis (Boyce 2004).



#### 4.4.2 Distribution and subtypes of mast cells

Mast cell development requires intact function of the membrane-bound growth factor termed stem cell factor (SCF) and its receptor, the tyrosine kinase Kit. Mice lacking function in either SCF or Kit are profoundly deficient in all subtypes of mast cells. WBB6F1 mice, which were used in the present work, lack mast cells due to a mutation in the Kit oncogene of SCF receptors. Mast cells originate from hematopoietic CD34<sup>+</sup> c-kit<sup>+</sup> progenitor cells in the bone marrow. When released and migrated into the extravascular space, mast cells develop *in situ* from these progenitors into two distinct mast cell subpopulations dependent on the microenvironments of tissues (Boyce 2004). Distribution of mast cells is diversified in different organs and tissues as well as in between different species. For instance, mast cells can not be found in the mouse liver but are abundant in the dog liver (Gersch *et al* 2002). Mature tissue mast cells or connective tissue mast cells (CT) are commonly found in connective tissues while mucosal mast cells or reactive mast cells are abundant in the gastrointestinal tract during helminthes infection (Boyce 2004).

Alcian blue safranin staining may distinguish connective tissue type mast cells (CTMC) from mucosal tissue mast cells (MMC). The CTMC are stained in red and MMC turn blue. The mast cells of skeletal muscle shows red staining. (Gersch *et al* 2002).

Some major characters of two mouse mast cells are listed as (Table 3) (Feger *et al* 2002; Shimizu *et al* 2001).

#### 4.4.3 Activation of mast cells

The classical way of mast cell activation is immunological and IgE-mediated. The receptors named FcεRI on the mast cell surface are aggregated by the binding of IgE and multivalent antigen or allergens. Receptor cross-linking may activate intracellular signal transduction including activation of serine proteases, tyrosine phosphorylation of multiple cellular substrates, increase of c-AMP and Ca<sup>2+</sup> levels.

Table 3. Major characteristics of the two mouse mast cell subtypes

	<b>Mucosal tissue</b>	<b>Connective tissue</b>
<b><i>Morphology &amp; biochemistry</i></b>		
Alcian blue safranin staining	Blue	Red or mixed
Granules	Small, variable	Large, uniform
Heparin	No	Yes
Tryptase	No	Yes
Chymase	Yes	Yes
Histamine	Yes	Yes
Serotonin	No	Yes
<b><i>Degranulation</i></b>		
IgE	+++	++
Compound 48/80	+	+++

+++ the maximal release of mediators

++ sub-maximal release of mediators

+ limited release of mediators

It provokes cytoskeleton rearrangement and subsequent fusion of cytoplasm granules and the cell membrane, finally resulting in mast cell degranulation and the generation of various inflammatory mediators (Schramm and Thorlacius 2004). Mast cells can be activated by non-immunological ways as well, such as physical stress, trauma and cold, ischemia/reperfusion-induced oxidant production, complement split products, the neuropeptide substance P and compound 48/80. Compound 48/80 is another classic activator of mast cells. Activation of mast cells triggered by compound 48/80 is similar to that of IgE, and both cause a similar secretory response including histamine release (Paton 1951).

Moreover, there are many other receptors expressed on the surface of mast cells, such as toll-like receptors and chemokine receptors (Table 2.). Mast cells have

capacity to respond to the ligands of these receptors. It has been reported that mast cells are activated by MCP-1, a CC chemokine (Alam et al 1994). Toll-like receptors on mast cell surface mediate the production of proinflammatory cytokines and chemokines (Takeda *et al* 2003, Matsushima *et al* 2004). As mentioned above, mast cells express several chemokine receptors, indicating that mast cells have the ability to respond to chemokines. Such activation without overt degranulation may be associated with selective release of specific mediators from the mast cells (Matsushima *et al* 2004, Theoharides and Cochrane 2003). Therefore, mast cell activation is not only involved in allergic reactions but also to acute, chronic, nonspecific and innate inflammation as well as adaptive immunity.

#### 4.4.4 Mediators of mast cells

Activated mast cells release many pro-inflammatory mediators stored in granules, including histamine, proteases, chemokines and TNF- $\alpha$  and newly synthesized lipid mediators, such as leukotrienes, prostaglandins and pro-inflammatory cytokines are synthesized and secreted as well.

When mast cells are activated immunologically or by compound 48/80, histamine stored in granule is released. Histamine has various effects in the inflammatory reaction including increased vascular permeability, vasodilatation, and contraction of respiratory and gastrointestinal smooth muscle and provoked mucus secretion. Histamine is the key mediator in IgE-dependent hypersensitivity (allergic) reaction. The histamine effects on the local vascular bed are mainly mediated by the H1-receptor. Histamine has ability to activate endothelial cells to express P-selectin within minutes both *in vitro* and *in vivo*. Moreover, histamine was able to increase leukocyte rolling but this rolling can be abolished by H1-receptor antagonist (diphenhydramine) (Asako *et al* 1994, Yamaki *et al* 1998b). Thus, the H1-receptor mediates leukocyte rolling induced by histamine. Other important mediators are leukotrienes (LTB<sub>4</sub>, Cys-LT). Leukotrienes are synthesized from membrane-associated arachidonic acid mainly by 5-lipoxygenase. LTB<sub>4</sub> is a potent

chemoattractant and induces neutrophil extravasation (Spada *et al* 1997). In addition, cysteinyl LTC<sub>4</sub>, LTD<sub>4</sub> and LTE<sub>4</sub> have been shown to augment microvascular permeability, thereby enhance extravasation of leukocytes from the circulatory system (Leng *et al* 1988). Potent and specific leukotriene biosynthesis inhibitors such as MMK-886 or A-63162 can inhibit leukocytes adhesion *in vivo* (Ramos *et al* 1991).

Mast cells are the only cell type capable of storing pre-synthesized TNF- $\alpha$  *in vivo* (Gordon *et al* 1990). Thus mast cells provide the only ready available source of TNF- $\alpha$  within peripheral tissues during the early course of infection. Indeed, mast cells secreted TNF- $\alpha$  within minutes of bacterial challenge and induced neutrophil recruitment to mediate bacteria clearance (Zhang *et al* 1992; Malaviya *et al* 1996; Echtenacher *et al* 1996). Chronic synthesis and release of TNF- $\alpha$  derived from mast cells can be recognized as an important factor in promoting inflammatory chronicity (Gordon *et al* 1991). Indeed, chemokines including CC and CXC may be produced by activated mast cells as well (Schramm and Thorlacius 2004).

#### 4.5 Glucocorticoids

Glucocorticoids are steroid hormones. They became the most potent drugs for acute and chronic inflammation diseases in clinic medicine in the late 1940s. The glucocorticoids are used on the allergic, autoimmune disease and leukemia and lymphomas as well as in preventing graft rejection after organ transplantation. But glucocorticoids have many severe side effects such as osteoporosis, atrophy of the skin, disturbance of metabolism, possibility of bacterial infection due to depressed immune function. One result is that use of glucocorticoids is often limited although it may exert beneficial anti-inflammatory effects in patients.

The clinical effect of glucocorticoids is dependent on two pharmacological properties. The first action is anti-inflammatory and involved the repression of inflammatory mediators and the synthesis of anti-inflammatory molecules. The second one is the induction of leukocyte apoptosis, predominantly T cell

(Tuckermann *et al* 2005). The molecular mechanism of glucocorticoids is similar to all steroid hormones. Specific binding of glucocorticoids with its receptor results in release of the chaperones and translocation into the nucleus, in which the complex binds as a homodimer to glucocorticoid response elements (GRE) on the DNA to regulate target gene expression. Besides, it can interact with other transcription factors such as nuclear factor- $\kappa$ B (NF- $\kappa$ B) and inhibit its translocation or function (Schramm and Thorlacius 2004).

Glucocorticoids, especially dexamethasone, play an anti-inflammatory role in mast cell-dependent reactions. The anti-inflammatory mechanism of glucocorticoids is not completely understood yet. It was reported that dexamethasone can't decrease the release of histamine, leukotrienes and prostaglandins in mast cells challenged by IgE (Cohan *et al* 1989). Moreover, glucocorticoids seems not to alter P-selectin-mediated rolling of leukocytes following local challenge with compound 48/80, suggesting that selectin expression is not a target of dexamethasone in mast cells-dependent leukocyte recruitment (Schramm *et al* 2002). On the other hand, it was found that dexamethasone has ability to inhibit firm adhesion of leukocytes to endothelial cells (Zhang and Thorlacius 2000). It has been shown that CXC chemokine formation and release is a key target of dexamethasone in mast cell dependent leukocyte recruitment (Schramm *et al* 2002). It has been reported that neutrophil recruitment induced by MIP-2 may be decreased by dexamethasone as well but not when stimulated by KC. Dexamethasone may prevent the extravasation of neutrophils by reducing expression of cell adhesion molecules on endothelial cells and leukocytes (Mori *et al* 1999). Considering the importance of chemokine-dependent leukocyte recruitment in pathological inflammation, it is necessary to clarifying the effect of glucocorticoids on TNF- $\alpha$ -induced chemokine production and function on endothelial cells.

## 5. AIMS OF THE THESIS

**I.** To examine the effect of TNF- $\alpha$  on the gene and protein expression of MIP-2 in endothelial cells and effect of dexamethasone on TNF- $\alpha$ -induced expression of MIP-2 and adhesion of neutrophils to endothelial cells.

**II.** Investigate the effect and mechanism of CC (MIP-1 $\alpha$ , MCP-1) and CXC (MIP-2, KC) chemokines on the extravasation process of neutrophils *in vivo* and the potential role of endothelial selectins (P- and E-selectin) in leukocyte recruitment induced chemokines.

**III.** Examine the role of mast cells in neutrophil recruitment induced by chemokines *in vivo*.

## 6. MATERIALS AND METHODS

### 6.1. Animals

Male C57/BL/6, TNF- $\alpha$  deficient, WBB6F1 (Jackson Laboratory, Bar Harbor, ME, USA) mice weighing 23-26 g were maintained on 12-h dark and 12-h light cycles and given food and water *ad libitum*. WBB6F1 mice are mast cell deficient due to a disruption in the Kit oncogene on chromosome 5, encoding the tyrosine kinase receptor for stem cell factor (SCF), which is essential for the development of mast cells. The animal experiments were approved by the regional Ethical Committee for Animal Experimentation. Mice were anaesthetized with 7.5 mg ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 2.5 mg xylazine (Janssen Pharmaceutical, Beerse, Belgium) per 100 g body weight *i.p.* Blood samples were taken from the tail artery after the experiment for analysis of systemic and differential leukocyte counts using a hemocytometer.

### 6.2. Cells

#### 6.2.1. Bone marrow mast cell (BMMC)

Mice were sacrificed and intact femurs and tibiae were removed. Sterile medium without FCS was repeatedly flushed through the bone shaft using a needle and 1 ml syringe, and the bone marrow cells were passed through a sterile wire screen to remove any bone fragments.

The cell suspension was centrifuged at 800 rpm for 20 min at 4° C and then cultured at a concentration of  $0.5-1 \times 10^6$  cells/ml in RPMI 1640 with 10% FCS, 50  $\mu$ M 2-mercaptoethanol and 25% (V/V) WEHI cell conditional medium as a source of IL-3, (BMMC medium). After 3 weeks, the SCF 40 ng/ml was added in the above culture medium.

The BMMC medium was replaced 3 times a week with fresh BMMC medium. Bone marrow mast cells were monitored for purity after 4 weeks by 1% Toluidine blue staining of fixed cytocentrifuge preparations. Once the BMMC population reached purity > 99% (5-10 weeks), they were used in subsequent experiments.

#### 6.2.2. Neutrophil isolation

Neutrophils were freshly isolated from C57/BL/6 mice. The bone marrow was flushed aseptically out of the femurs and humeri bones with ice-cold PBS and then neutrophils were isolated by using Ficoll-Paque<sup>TM</sup> Research Grade/Amersham Pharmacia Biotech, Uppsala, Sweden). The purity of bone marrow neutrophils was higher than 70% as assessed by Turk stain in a hemocytometer. Neutrophils were resuspended in culture medium until use in the adhesion assay.

#### 6.2.3. Endothelial cells

The polyoma transformed murine endothelioma cell line eEnd.2 was cultured in DMEM supplemented with 10% fetal calf serum, L-glutamine, penicillin and streptomycin and subcultures twice weekly as described previously (Williams *et al* 1989).

### 6.3. Intravital microscopy

The cremaster muscle was prepared for intravital microscopy. In brief, a midline incision of the skin and fascia was made over the ventral aspect of the left scrotum and the incised tissues were retracted to expose the cremaster muscle sack. The cremaster muscle was then separated from the epididymis and the testis was pushed back to the side of the preparation. The preparation was performed on a transparent pedestal to allow transillumination and a microscopic observation of the cremaster muscle microcirculation was undertaken after a 15-min equilibration time. Intra-scrotal injection of chemokines (R & D Systems Europe, Ltd., Abingdon, Oxon, U.K.) or other drugs (fMLP, TNF- $\alpha$ ) at indicated doses, diluted



in 0.15 ml phosphate-buffered saline (PBS) was performed at 3 hr prior to microscopic observation. In order to delineate the role of the selectins in chemokine-induced neutrophil recruitment, monoclonal antibodies to P-selectin or to E-selectin or control antibody (Pharmingen, San Diego, CA, U.S.A.) were given i.v. immediately and individually prior to intra-scrotal administration of chemokines. In separate experiments, the role of histamine and leukotrienes in chemokines-induced leukocyte recruitment was evaluated in mice pretreated individually with the H1-receptor antagonist diphenhydramine hydrochloride 10 mg/kg (a dose that abolishes histamine-induced leukocyte rolling) and the leukotriene biosynthesis inhibitor MK-886 by oral administration with 1.0 mg/kg twice a day for 2 days with a final dose 10 min before surgery (Sigma Chemical Co., St. Louis, MO, U.S.A.). Observations of the cremaster microcirculation were made using an Olympus microscope (BX50WI, Olympus Optical Co. GmbH, Hamburg, Germany) equipped with water immersion lenses (x40/NA 0.75 and x63/NA 0.90). The microscopic image was televised (Sony Triniton) using a charge-coupled device video camera (FK 6990 Cohu, Pieper GmbH, Berlin, Germany) and recorded on videotape (Panasonic SVT-S3000 s VHS recorder) for subsequent off-line analysis. Analyses of leukocyte flux and leukocyte-endothelium interactions (rolling and adhesion) were made in venules (inner diameter 30-38  $\mu\text{M}$ ) with stable resting blood flow.

Rolling leukocyte flux was determined at indicated time points by counting the number of rolling leukocytes per 30 s passing a reference point in the microvessel and expressed as cells per min. Leukocyte adhesion in venules (stationary for  $\geq 30$  s) was counted along 680  $\mu\text{M}$  long segments of the endothelial lining on one side of the vessel lumen and expressed as number of adherent cells per mm. Red blood cell velocity was measured on-line by the use of an optical Doppler velocimeter (Microcirculation Research Institute, Texas A & M University, College Station TX, U.S.A.). Venular wall shear rate was determined based on the Newtonian definition: wall shear rate = 8 (red blood cell velocity

/1.6)/venular diameter) as described previously (House and Lipowsky 1987).

#### 6.4. Histology

Samples of intact cremaster muscle microvascular networks were fixed in 4% formaldehyde over night and then stained with Giemsa stain for 1h. After differentiation in acetic acid (0.01%) for 10 min, the samples were mounted on gelatin pre-coated glass slides and covered with a cover glass as described in detail previously for the rat mesentery (Yamaki *et al* 1998a). Leukocyte emigration was quantified by counting the number of extravascular polymorphonuclear (PMNL) and mononuclear (MNL) leukocytes per high power field observed along a randomly selected venous in each preparation and expressed as the number of cells per mm<sup>2</sup>.

#### 6.5. Cell adhesion assay

Endothelial cells were plated at a density of  $2 \times 10^4$  cells per well in 96-well plates. When confluent, the cells were stimulated with wild type or TNF- $\alpha$  deficient BMMC incubated with or without MIP-2 or compound 48/80 for 4 hours, and then  $2 \times 10^4$  neutrophils were added into each well and incubated for 30 min. Subsequently, the wells were washed with PBS three times in order to remove nonadherent cells. Next 50  $\mu$ l 0.005 M phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (HTAB) was added into each well followed by 150  $\mu$ l of 3,3',5,5',- tetramethylbenzidine liquid substrate system and incubated for 10 min at 25 °C for color development. At the end, 100  $\mu$ l of 0.5 M of H<sub>2</sub>SO<sub>4</sub> was added in order to stop the oxidation reaction. The enzyme activity was determined spectrophotometrically as the myeloperoxi-dase-catalyzed change in absorbance occurring in the redox reaction of H<sub>2</sub>O<sub>2</sub>-tetramethylbenzidine (450 nm, 25 °C). A standard curve was constructed using defined quantities of neutrophils in the same plate.

### **6.6. Flow cytometry**

Endothelial cells were subcultured in 12-well plates ( $2 \times 10^5$  cells per well) for 48 h subsequently incubated with 100 ng/ml of TNF- $\alpha$  (R&D system Europe), MIP-1 $\alpha$  and MCP-1 for 3 h at 37° C. Endothelial cells were isolated by trypsination (0.1%) and washed with PBS (centrifuge 150 x g) for 5 min. Then endothelial cell samples were stained with a FITC-labeled rat anti-mouse P-selectin (RB40.34, 1  $\mu$ g per  $10^6$  cells) or a FITC-labeled isotype-match control antibody for 20 min at room temperature. The cell pellet was re-suspended with 0.5 ml PBS and put on ice until analysis, which was performed within 30 min. The intensity of P-selectin expression is given as the mean fluorescence intensity of endothelial cells incubated with the anti-P-selectin antibody divided by the mean fluorescence intensity of cells incubated with the control antibody.

### **6.7. RT-PCR**

Total RNA was extracted from samples using an acid guanidinium-phenol-chloroform method (TRIzol Reagent; GIBCO-BRL, Life Technologies, Grand Island, NY, U.S.A.) and treated with RNase-free DNase (DNase I; Amersham Pharmacia Biotech Sollentuna Sweden) in order to remove potential genomic DNA contaminants according to manufacturer's protocol. RNA concentrations were determined by measuring in the absorbance at 260 nm spectrophotometrically. RT-PCR was performed with SuperScrip One-Step RT-PCR system (GIBCO-BRL Life Technolgies, Grand Island, NY. U.S.A.). Each reaction contained 500 ng of cellular total RNA as a template and 0.2  $\mu$ M of each primer a final volume of 50  $\mu$ l. Mouse  $\beta$ -actin served as an internal control gene. The RT-PCR profile was one cycle of cDNA synthesis at 50° C for 30 min, followed by 35 cycles of denaturation at 94° C for 30 s, annealing at 55° C for 30 s and extension at 72° C for 1 min. one cycle of final extension at 72° C for 10 min. After RT-PCR,

aliquots of the RT-PCR products were separated using electrophoresis on a 2% agarose gel containing ethidium bromide and photographed. The primers sequences of MIP-2, P-selectin, E-selectin and  $\beta$ -actin were as shown in the following Table 4.

Table 4

<b>Primers</b>	<b>Sequence</b>
$\beta$ -actin (f)	5'-ATG TTT GAG ACC TTC AAC ACC-3'
(r)	5'-TCT CCA GGG AGG AAG AGG AT-3'
MIP-2 (f)	5'-GCT TCC GGC ACT CCA GAC-3'
(r)	5'-TTA GCC TTG CCT TTG TTC AGT AT-3'
P-selectin (f)	5'-ACG AGC TGG ACG GAC CCG -3'
(r)	5'-GGC TGG CAC TCA AAT TTA CAG-3'
E-selectin (f)	5'-GGT AGT TGC ACT TTC TGC GG-3'
(r)	5'-CCT TCT GTG GCA GCA TGT TC-3'
CXCR2 (f)	5'-TGT TCT TTG CCC TGA CCT TGC-3'
(r)	5'-ACG CAG TAC GAC CCT CAA ACG-3'.

### 6.8. ELISA

Culture medium from the endothelial cells or endothelial cells with BMMC stimulated individually by reagents was collected, the level of TNF- $\alpha$ , MIP-2 in cell culture supernatant was determined using a double-antibody-specific Quantikine ELISA kit (R&D Systems Europe. Ltd., Abingdon. Oxon, UK). The standard curve was constructed using recombinant murine TNF- $\alpha$  or MIP-2 (R&D

Systems Europe). The minimum detectable concentration of TNF- $\alpha$  is in this assay less than 5.1 pg/ml and MIP-2 is 20 pg/ml.

#### **6.9. Statistic analysis**

Data are given as mean values  $\pm$ SEM and  $n$  represents the number of samples or animals per experimental group. Statistical evaluations were performed using Kruskal-Wallis one-way ANOVA on Ranks (Dunnetts method) for unpaired samples. Differences between different treatments and/or groups were considered significant at values of probability less than 0.05.

## 7. RESULTS AND DISCUSSION

### *MIP-2 expression in endothelial cells activated by TNF- $\alpha$*

TNF- $\alpha$  plays a key role in leukocyte recruitment *in vivo* and in adhesion of endothelial cells to neutrophils *in vitro* as well. Firm adhesion to endothelium is a critical step in the extravasation process of neutrophils and may result from the mediators, such as chemokines, released from these activated cells. CXC chemokines (MIP-2 and KC) have the ability to attract and activate neutrophils. The objective of this study was to examine the effect of TNF- $\alpha$  on gene and protein expression of MIP-2 in endothelial cells. Total RNA was isolated from endothelial cells, reverse transcribed into cDNA and PCR amplified with specific primers for MIP-2. It was found that TNF- $\alpha$  (100 ng/ml) with 1 h stimulation induced clear-cut expression of MIP-2 mRNA in endothelial cells. In contrast, the MIP-2 mRNA signal was not detectable in untreated control cells. By ELISA, it was also observed that challenge with TNF- $\alpha$  increased MIP-2 expression in the endothelial cells in a dose- and time-dependent fashion.

Previous studies on the cellular source of MIP-2 in TNF- $\alpha$ -induced inflammation have implicated that macrophages, fibroblasts and epithelial cells are capable of expressing MIP-2 (Driscoll *et al* 1993). Thus, our novel data expand on previous findings by demonstrating that endothelial cells also express MIP-2 at the gene and protein level in response to stimulation of TNF- $\alpha$ . Moreover, in the assay of adhesion between neutrophils and endothelial cells, baseline adhesion of neutrophils was  $16 \pm 2$  cells/mm<sup>2</sup>, and after 2 h exposure of endothelial cells to 100 ng/ml of TNF- $\alpha$ , neutrophil adhesion increased more than three times to  $51 \pm 6$  cells/mm<sup>2</sup>. Interestingly, addition of a monoclonal antibody against MIP-2 (2.5  $\mu$ g/ml) reduced TNF- $\alpha$  induced neutrophil adhesion to almost baseline ( $17 \pm 2$  cells/mm<sup>2</sup>). It suggests that MIP-2 secreted by endothelial cells is an important mediator of neutrophil adhesion in response to TNF- $\alpha$  stimulation. This notion is in

line with a recent *in vivo* study showing that dermal accumulation of neutrophils provoked by TNF- $\alpha$  is markedly reduced by passive immunization against MIP-2 (Tessier *et al* 1997). We demonstrated that TNF- $\alpha$  can activate directly endothelial cells and produce MIP-2, which is an important mediator of adhesion to neutrophils *in vitro*.

Glucocorticoids constitute a common therapy against inflammatory disease. These potent anti-inflammatory agents, including dexamethasone, have been shown to effectively inhibit the inflammatory cell response (Schleimer *et al* 1993). In spite of the fact that dexamethasone have been demonstrated to attenuate TNF- $\alpha$ -induced leukocyte adhesion and extravascular recruitment, the detailed mechanisms by which dexamethasone inhibits tissue accumulation of leukocytes remain elusive. Interestingly, previous studies have shown that dexamethasone inhibits gene and protein expression of KC (Deng *et al* 1994). However, the literature on the effect of dexamethasone on MIP-2 expression is rather complex and partly contradictory. For example, it has been shown that *in vivo* treatment with dexamethasone has no effect on MIP-2 production while others have reported that MIP-2 expression is reduced by dexamethasone (O'Leary *et al* 1997; Yi *et al* 1996).

We wanted to determine the effect of dexamethasone on TNF- $\alpha$ -induced expression of MIP-2 and adhesion of neutrophils to endothelial cells. Pretreatment of endothelial cells with 0.01 and 1  $\mu$ M of dexamethasone for 2 h decreased MIP-2 protein expression in response to TNF- $\alpha$  (1–100 ng/ml). This reduction in MIP-2 expression exerted by dexamethasone (1  $\mu$ M) corresponded to an 84% and 37% decrease in the case of 1 and 100 ng/ml of TNF- $\alpha$  challenge, respectively.

The literature on the impact of dexamethasone on the regulation of MIP-2 is complex and partly contradictory. For example, one group has reported that dexamethasone treatment has no effect on LPS-induced expression of MIP-2 in the lung (O'Leary *et al* 1997) whereas others can not confirm such findings (Yi *et al* 1996). Moreover, another study has shown that dexamethasone inhibits MIP-2

expression provoked by ozone (Haddad *et al* 1995). Our observations support the concept that MIP-2 belongs to the list of chemokines that is negatively regulated by dexamethasone, including KC, MIP-1a and MCP-1 (O'Leary *et al* 1997, Taylor *et al* 1999, Kawahara *et al* 1999). In the present study, we could also confirm that dexamethasone almost completely inhibited neutrophil adhesion to endothelial cells stimulated with TNF- $\alpha$ . The detailed mechanisms by which glucocorticoids inhibit tissue accumulation of leukocytes remain complex and incompletely understood. A study has shown that glucocorticoids downregulates mediator-induced expression of endothelial adhesion molecules, such as E-selectin and ICAM-1 (Cronstein *et al* 1992). On the other hand, some investigations have reported that dexamethasone primarily attenuates the expression of CD18 on the surface of leukocytes. Our data expand on these findings and suggest that inhibition of MIP-2 expression in endothelial cells may be a fundamental mechanism of action explaining the attenuated inflammatory cell response exerted by dexamethasone. Such a mechanism could also explain the reduced CD18 expression on leukocytes observed following administration of glucocorticoids (Davenpeck *et al* 1998; Burton *et al* 1995), considering the fact that one of the main functions of CXC chemokines is to activate neutrophils in which increased expression of CD18 is a key feature. Thus, our data suggest that dexamethasone is a negative regulator of MIP-2 expression in TNF- $\alpha$  activated endothelial cells and that this mechanism of action may help explain, at least partly, the inhibitory effect of glucocorticoids on neutrophil recruitment in inflammatory reactions.

*Chemokines can induce all steps of the neutrophil recruitment process (rolling, adhesion, and migration) in vivo.*

It was found that challenge with both CC chemokines (MIP-1 $\alpha$  and MCP-1) and CXC chemokines (MIP-2 and KC) induced all steps in the leukocyte extravasation process, including rolling, adhesion and extravascular accumulation in a dose-dependent manner. At 300 ng of CC chemokines, rolling leukocytes flux was



increased by more than 2-fold and adherent and extravascular neutrophils by more than 6-fold compared with normal control. This suggests that CC chemokines are capable of inducing clear-cut neutrophil recruitment *in vivo*. However, the CXC chemokine MIP-2 was found to be much more effective in stimulating infiltration of neutrophils. For example, 30 ng of MIP-2 caused similar neutrophil response as 300 ng of MIP-1 $\alpha$  and MCP-1. Both extravascular and venular leukocytes in the cremaster muscle microcirculation are almost exclusively neutrophils. On the other hand, it was found that fMLP, a typical chemoattractant has no ability to induce leukocyte recruitment in the undisturbed cremaster muscle tissue *in vivo*.

A common held view in chemokine biology is that CXC chemokines typically regulate neutrophil trafficking and that CC chemokines predominantly coordinate movement of mononuclear leukocytes. Our present studies challenge that concept and provides evidence demonstrating that CC chemokines, MIP-1 $\alpha$  and MCP-1, have the capacity to provoke neutrophil recruitment *in vivo*. Moreover, the present investigation demonstrates that both CXC and CC chemokines possess the ability to induce all steps in the extravasation process, including rolling, adhesion as well as recruitment of neutrophils *in vivo*. In contrast, most studies on chemotaxis *in vitro* have show that CC chemokines do not have the capacity to provoke neutrophil migration (Bonecchi *et al* 1999, McColl *et al* 1993, Cheng *et al* 2001). However, these artificial *in vitro* systems comprise only neutrophils with or without endothelial cells and neglect indirect effects mediated by other cells *in vivo*. Moreover, this is in contrast to our findings on fMLP, which is a prototype of classical chemoattractants, which had no effect on neutrophil infiltration *in vivo*. Thus, on the basis of these results, it is suggested that these chemokines act in a principally distinct manner from classical chemoattractants by which chemokines are capable of activating endothelial cells in tissues *in vivo*, but is very effective chemo-attractant for neutrophils *in vitro*. Our findings expand on previous studies by elucidating the detailed mechanisms of action of chemokines on leukocyte-endothelium interactions and may help clarify the complex mechanisms of action

of chemokines in acute inflammation, which apparently is not restricted to direct activation of neutrophils but also possesses other unknown effects *in vivo*.

*Chemokine-induced leukocyte recruitment is P-selectin-dependent.*

It has been demonstrated that TNF- $\alpha$ -induced neutrophil accumulation is critically dependent on P-selectin-mediated rolling (Månsson *et al* 2000). This observation indicated that leukocyte rolling supported by P-selectin on the vascular endothelium is a fundamental and common pathway for neutrophils.

To evaluate the role of the P-selectin in the neutrophil response to chemokine challenge, we pretreated animals with monoclonal antibodies directed against mouse P-selectin and E-selectin. Pretreatment with the P-selectin antibodies significantly reduced leukocyte rolling in CC chemokine treated venules by more than 95% and in CXC chemokine by more than 96%. Thus, chemokine-induced rolling of leukocytes was extremely dependent on P-selectin expression on the surface of endothelial cell. On the other hand, administration of the antibody against E-selectin had no effect on leukocyte-endothelium interactions (rolling, adhesion and recruitment) in response to stimulation with chemokines. The observation that E-selectin is not involved in the rolling adhesive process is in line with previous studies showing that blocking E-selectin has no effect on TNF- $\alpha$ -induced leukocyte rolling (Wan *et al* 2002) and that leukocyte rolling is intact in E-selectin-deficient mice (Milstone *et al* 1998). The role of E-selectin in the extravasation process of neutrophils remains elusive but may be involved in events downstream of rolling, such as activation and adhesion of rolling leukocytes (Milstone *et al* 1998, Ley *et al* 1998, Simon *et al* 2000). Interestingly, we observed that, in parallel to the reduction in rolling, inhibition of P-selectin function abolished the number of firmly adhered and extravascular neutrophils in response to chemokines challenge. This may suggest that P-selectin-dependent rolling is a prerequisite in chemokine-induced neutrophil adhesion and extravasation as well as indicating that chemokines have the ability to activate

vascular endothelial cells *in vivo*. In contrast, fMLP, a classical chemoattractant, had no effect on neutrophil infiltration in undisturbed tissues. It is suggested that chemokines act in a principally distinct manner different from classical chemoattractants in that these chemokines are capable of activating endothelial cells *in vivo*.

*Chemokine-induced neutrophil recruitment is mediated by mast cells in vivo.*

A key feature in the extravasation process of inflammatory cells is activation of endothelial cells. Activated endothelium expresses P-selectin, which has been documented to be of paramount importance in supporting neutrophil rolling and recruitment (Mansson *et al* 2000, Ley *et al* 1995, Robinson *et al* 1999). As previously described (Weller *et al* 1992), we observed that TNF- $\alpha$  was a potent inducer of P-selectin expression on the surface of isolated endothelial cells *in vitro*. In contrast, challenge with CC chemokines had no effect on the expression of P-selectin on endothelial cells. Moreover CXCR2 mRNA could not be detected in endothelial cells, meaning that endothelial cells can not directly respond to chemokines. On the other hand, our findings showing that chemokines trigger influx of neutrophils into the extravascular compartment, which was completely abolished by injection of the anti-P-selectin antibody, suggest that chemokines indirectly activate the vascular endothelial cells *in vivo*. Moreover, our data on gene expression in the cremaster muscle demonstrates that chemokines increase E-selectin mRNA levels, indicating that endothelial cells are indeed activated in response to chemokine challenge by some mediator or mechanism *in vivo*.

We used mutant mice to define the role of tissue mast cells in chemokine-induced neutrophil extravasation induced by MIP-1 $\alpha$  and MIP-2. It was found that the neutrophil extravasation provoked by MIP-1 $\alpha$  was significantly reduced in mast cell-deficient mice, *i.e.* the numbers of rolling, adherent and transmigrated neutrophil were markedly decreased 52%, 84% and 58% respectively. Similarly,

we found that MIP-2 provoked leukocyte rolling, adhesion and recruitment was reduced by 86%, 82% and 71%, respectively.

It is interesting that chemokine-induced neutrophil recruitment was greatly reduced in mice lacking mast cells, suggesting that chemokine-mediated endothelial cell activation and neutrophil responses are mediated via mast cells. Activated mast cells can release a number of substances including histamine, leukotrienes and TNF- $\alpha$  with the ability to increase P-selectin expression on endothelial cells and in turn promote neutrophil rolling and extravascular recruitment (Ley *et al* 1995, Yamaki *et al* 1998a, Kanwar *et al* 1995). In this study, we found that MIP-2-induced neutrophil recruitment was reduced in mast cell-deficient mice by more than 71% in cremaster muscle. Taken together, it may be forwarded that mast cells activation may constitute a common feature in both CC and CXC chemokine-induced infiltration of neutrophils *in vivo*. Moreover, we found clear-cut expression of CXCR2 mRNA in both PMNL and BMMC but not in endothelial cells using RT-PCR. These results support that CXC chemokine-induced PMNL recruitment *in vivo* could be mediated by mast cells acting as an intermediary cell in the extravascular tissue. In fact, this expression of CXCR2 on murine mast cells is in line with previous observations showing that human mast cells also express CXCR2 (Lippert *et al* 1998, Nilsson *et al* 1999).

#### *Endothelial cell activation*

Endothelial cell activation is a critical step in the extravasation process of neutrophil *in vivo* due to the important role of endothelial P-selectin expression in supporting leukocyte rolling (Weller *et al* 1992, Mansson *et al* 2000). In order to better understand the mechanisms of chemokine-induced leukocyte recruitment, we studied P-selectin expressed on the surface of endothelial cells stimulated by chemokines (MIP-1 $\alpha$  and MCP-1) and TNF- $\alpha$  *in vitro* using flow cytometry. It was found that that challenge with TNF- $\alpha$  (100 ng/ml) for 3 hr markedly increased the expression of P-selectin on the surface of endothelial cells but challenge with

CC chemokines (10-1000 ng/ml) failed to stimulate P-selectin expression on endothelial cells. Since P-selectin is expressed at baseline in Weibel-Palade bodies in endothelial cells (van Mourik *et al* 2002), mRNA expression of P-selectin is not an ideal indicator of endothelial cell activation *in vivo*. Moreover, P-selectin is not limited to endothelial cells as it is also expressed in platelets. In contrast to P-selectin, E-selectin is not expressed at baseline and the stimulated expression of E-selectin is limited to endothelial cells. Thus, E-selectin mRNA expression makes up an ideal indicator of endothelial cell activation in tissues *in vivo*. Indeed, we observed no expression of E-selectin mRNA in the cremaster muscle of negative control mice. However, challenge with TNF- $\alpha$  (positive control) and CC chemokines, MIP-1 $\alpha$  and MCP-1, significantly increased gene expression of E-selectin. As expected, P-selectin mRNA was detected at baseline and treatment with TNF- $\alpha$ , MIP-1 $\alpha$  and MCP-1 further enhanced levels of P-selectin mRNA in the cremaster muscle. Herein, we found that challenge with both CC chemokines (MIP-1 $\alpha$  and MCP-1) and CXC chemokine (MIP-2) increased gene expression of E-selectin (a marker of endothelial cell activation) in wild-type mice. Interestingly, this chemokine-induced expression of E-selectin was absent in mast cell-deficient mice, suggesting that endothelial cells could not be activated by chemokines in tissues lacking mast cells. Thus, these novel findings indicate that chemokine-induced activation of endothelial cells is mediated *via* mast cells *in vivo*. In contrast, TNF- $\alpha$  induced gene expression of E-selectin was intact in mast cell-deficient mice. This lead to the hypothesis that TNF- $\alpha$  derived from mast cells may be the intermediary mediator of chemokine-induced activation of endothelial cells *in vivo*.

*Mast cell-derived TNF- $\alpha$  mediates CXC chemokine-induced leukocyte recruitment.*

As mentioned and shown above, TNF- $\alpha$  is a very strong activator of endothelial cells with the capacity to up-regulate P- and E-selectin on the surface and produce chemokines in endothelial cells. It has been demonstrated that mast cells are an

important source of TNF- $\alpha$  due to a rapid and sustained release manner (Gordon *et al* 1991). In order to investigate a role of TNF- $\alpha$  in chemokine-induced neutrophil recruitment, we designed the following experiment. The classic chemoattractant fMLP alone does not induce leukocyte rolling (Lindbom *et al*, 1992) nor neutrophil recruitment in cremaster *in vivo*. Different doses of TNF- $\alpha$  (0.3-300 ng) were evaluated and it was found that 1.0 ng of TNF- $\alpha$  did not increase neutrophil recruitment but a higher dose (3.0 ng) provoked clear-cut neutrophil extravasation. Thus, based on these findings it was concluded that 1 ng of TNF- $\alpha$  act as a threshold dose of TNF- $\alpha$  with respect to triggering tissue accumulation of neutrophils. Interestingly, this threshold dose of TNF- $\alpha$  (1 ng) markedly potentiated fMLP-induced neutrophil recruitment in a dose-dependent manner. These findings indicate that fMLP indeed can induce neutrophil migration *in vivo* (when acting together with other mediators), but fMLP is dependent on the activation endothelial cells exerted by the threshold dose of TNF- $\alpha$ . These data indicate that TNF- $\alpha$  plays a critical role in terms of activating the endothelial cells resulting in P-selectin expression and leukocyte rolling, which chemokines and other chemoattractants such as fMLP are not able to do in a direct fashion. Thus, there is a complex interplay in tissues *in vivo* where endothelial cell activating factors, such as TNF- $\alpha$  constitute a critical precondition for subsequent firm adhesion and transmigration. In order to study the role of TNF- $\alpha$  in chemokine-induced neutrophil recruitment, the neutrophil recruitment induced by MIP-2 was performed both in mast cell and TNF- $\alpha$ -deficient mice group. Neutrophil extravasation induced by MIP-2 was significantly reduced by 97% in mast cell-deficient mice. Notably, we observed that neutrophil recruitment induced by MIP-2 was significantly reduced by 78% in TNF- $\alpha$ -deficient mice. In contrast, TNF- $\alpha$ -induced neutrophil recruitment was still intact in mast cell-deficient mice. It was found that WT and TNF- $\alpha$ -deficient mice contained similar levels of mast cells in cremaster muscle. It means that chemokine-provoked accumulation of neutrophils is dependent on or mediated by the presence of mast cells and TNF- $\alpha$ .

In order to determine whether CXC chemokine-induced neutrophil recruitment was mediated by TNF- $\alpha$  derived from mast cells or not, we adopted an *in vitro* adhesion assay as well. It was observed that MIP-2 increased the number of adherent neutrophils by more than 3-fold when co-incubation with wild-type mast cells. Interestingly, MIP-2-induced neutrophil adhesion was significantly decreased by 58% using TNF- $\alpha$ -deficient mast cells. Compound 48/80, a positive control, provoked neutrophil adhesion which was reduced by more than 60% using mast cells from TNF- $\alpha$  gene-targeted mice. These findings strongly suggest that chemokine-regulated neutrophil recruitment is mediated by mast cell-derived TNF- $\alpha$ . In separate experiments, mast cells stimulated with MIP-2 and compound 48/80 significantly increased TNF- $\alpha$  secretion by 71% and 225% respectively, showing that MIP-2 challenge, indeed, increase TNF- $\alpha$  secretion from mast cells. This finding helps explain the mechanism behind chemokine-induced neutrophil recruitment *in vivo* and elucidate an important part of the complex interactions between chemokines, tissue resident cells and circulating leukocytes.

*No role of histamine and leukotrienes in chemokine-provoked neutrophil recruitment.*

There are numerous different mediators, such as histamine, leukotrienes and cytokines, which may be released from mast cells upon stimulation. Histamine is known to provoke P-selectin expression and leukocyte rolling (Asako *et al* 1994, Yamaki *et al* 1998b). To evaluate the role of histamine in chemokine-induced leukocyte recruitment, animals were pretreated with the H1-receptor antagonist diphenhydramine (10 mg/kg), which effectively blocks histamine-provoked leukocyte rolling. However, inhibition of H1-receptors had no effect on chemokine (MIP-1 $\alpha$  and MIP-2)-induced neutrophil rolling, adhesion and recruitment, suggesting that histamine is not a likely candidate for mediating endothelial cell activation in chemokine-regulated accumulation of leukocytes. Leukotrienes can act as leukocyte chemoattractants as well. It has been reported

that MCP-1 indirectly provokes neutrophil recruitment peritoneal via production of leukotriene B4 (Matsukawa *et al* 1999). In this experiment, MK-886, a leukotriene biosynthesis inhibitor, given 2 days prior (1 mg/kg p.o.) had no effect on MIP-2-induced neutrophil recruitment, suggesting that leukotrienes are neither critical in CXC chemokine-induced neutrophil recruitment.

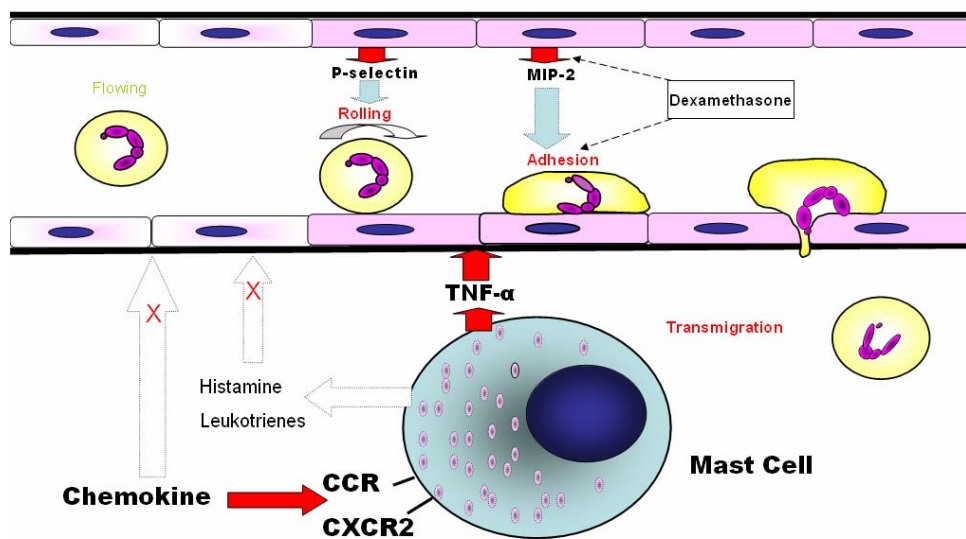


## 8. Summary (Swedish)

Inflammation är vävnadens svar på yttre störning och syftar till att eliminera främmande ämnen såsom bakterier men under vissa omständigheter initieras den inflammatoriska reaktionen av ofarliga substanser eller för starkt mot patogener och kan därmed orsaka vävnadsskada och är en underliggande mekanism vid många vanliga sjukdomar inkluderande allergier, blodförgiftning och inflammatorisk tarmsjukdom. Den viktigaste komponenten i den inflammatoriska reaktionen är vävnadsinfiltrationen av leukocyter. Rekryteringen av leukocyter är en flerstegsprocess som medieras av specifika adhesionsmolekyler som är uttryckta på kärlväggen och leukocyterna i samband med inflammation. En relativt ny grupp av *kemotaktiska cytokiner*, s.k. kemokiner stimulerar vävnadsmigration av leukocyter. Avhandlingen har studerat mekanismerna bakom kemokin-inducerad leukocytrekrytering. En central del i rekryteringen av leukocyter är aktivering av endotelceller som i sin tur uppreglerar P-selectin som medierar leukocytrullning i blodkärlen vilket är nödvändigt för den senare adhesionen och vävnadsackumuleringen av leukocyter. Avhandlingen har visat att kemokiner kan aktivera endotelceller *in vivo* (men inte *in vitro* eftersom receptorerna saknas på endotelceller) via aktivering av mast celler i vävnaden, vilka uttrycker kemokin receptorerna. Avhandlingen har också kunnat visa att det är mast cell deriverat TNF- $\alpha$  som medierar kemokin-inducerad endotelcells aktivering och P-selektin uttryck *in vivo*. Tidigare har man ansett, baserat på *in vitro* försök med endast endotelceller och leukocyter, att CC kemokiner stimulerar rekrytering av mononukleära leukocyter men inte neutrofiler. På samma sett har man ansett att CXC kemokiner huvudsakligen stimulerar neutrofiler men inte mononukleära celler. Dessa slutsatser har baserats på *in vitro* metoder som uteslutit andra vävnadsceller som bidrar till komplexiteten *in vivo*. Den här avhandlingen har visat att det inte är så enkelt och polariserat *in vivo* utan att både CC och CXC kemokiner effektivt stimulerar rekryteringen av neutrofiler.

Anledningen är att mast celler i vävnaden uttrycker receptor för både CC och CXC kemokiner vilket leder till endotelcells aktivering och därmed leukocytrekrytering. Dessa fynd bidrar inte bara till en fördjupad förståelse för inflammatoriska processer utan kan också vara viktiga för framtagandet av mer specifika och effektiva substanser riktade mot patologisk inflammation där kemokiner spelar en roll i patofysiologin.

Figure 3. The possible mechanism of chemokine-induced leukocyte recruitment *in vivo*



## 9. CONCLUSIONS

1. TNF- $\alpha$  is able to activate endothelial cells to express CXC chemokines and induce adhesion of neutrophil to endothelial cells. The pharmacological role of dexamethasone may be partly due to the inhibition of the chemokine secretion.

2. Chemokines, both CC (MIP-1 $\alpha$ , MCP-1) and CXC (MIP-2, KC), can induce all steps of the leukocyte recruitment process (rolling, adhesion, and transmigration) *in vivo*.

3. Leukocyte recruitment induced by chemokines is dependent on P-selectin-mediated leukocyte rolling.

4. Chemokine-induced endothelial cell activation and leukocyte recruitment are mediated by mast cell *in vivo*.

5. Mast cell-derived TNF- $\alpha$  plays a key role in chemokine-induced leukocyte recruitment via endothelial cell activation *in vivo*.

## 10. ACKNOWLEDGMENT

This work has been conducted at the Department of Surgery and Department of Experimental Research, Malmö University Hospital.

I would like to express my sincere gratitude towards:

My PhD study supervisor, Associate Professor, Henrik Thorlacius MD, PhD. He promoted me as his PhD student and let me have a regular and Western PhD training chance. I admire his vast knowledge and scientific skills. I appreciate his ambition to overcome any problems in medical research he met. Without his great scientific guidance and help, this thesis would not have been finished. Tack så mycket.

Special thanks to Professor Bengt Jeppsson, assistant dean of the medical faculty and chairman of the surgery department. He was very supportive, understanding and approachable for my PhD study and is very kind to help every foreign student. Tack så mycket.

Professor Lars-Magnus Bjursten. As the head of the Department of Experimental Research, my working place, and an opponent in half-time control of my PhD study, he gave me a lot of support in my study. Tack så mycket.

I hold Mrs. Anita Alm and Professor Robert Jarvella in high regard, they are always very kind to help me whenever I met problems. Tack så mycket.

My co-workers Dr. Mingxiu Wan, Dr. Li Xiang, Dr. Diya Adawi, Mrs. Susanne Eiswohld, Dr. Andrada Mihaescu, Mr. Muhammad Asaduzzaman. Dr. Amjid Riaz, Dr. Stefan Santén, Dr. Daniel Klintman, Dr Qi, Dr Chai, and all of the staffs and colleagues of the Department of Experimental Research and Department of Surgery in MAS. They are all my good friends and gave me a lot of help. Tack så mycket.

## 11. REFERENCES

- Alam R, Kumar D, Anderson-Walters D, Forsythe PA. Macrophage inflammatory protein-1 alpha and monocyte chemoattractant peptide-1 elicit immediate and late cutaneous reactions and activate murine mast cells in vivo. *J Immunol* (1994) **152**: 1298-1303
- Appelberg R. Macrophage inflammatory proteins MIP-1 and MIP-2 are involved in T-cell-mediated neutrophil recruitment. *J Leuk Biol* (1992) **52**: 303-306
- Asako H, Kurose I, Wolf R, Defrees S, Zheng ZL, Phillip ML, Paulson JC & Granger DN. Role of H1 receptors and P-selectin in histamine-induced leukocyte rolling and adhesion in postcapillary venules. *J Clin Invest* (1994) **93**: 1508-1515
- Bacon KB, Oppenheim JJ. Chemokines in disease models and pathogenesis. *Cytokine Growth Factor Rev* (1998) **9**: 167-173
- Baggiolini M, Dewald B, Moser B. Chemokines, Inflammation: Basic Principles and Clinical Correlates 3rd ed., edited by Gallin JI and Snyderman R. (1999) Chapter **28**: 419-431
- Bevilacqua MP, Pober JS, Mendrik DL, Cotran RS, Gimbrone MA, Jr. Identification of an inducible endothelial-leukocyte adhesion molecule. *Proc Natl Acad Sci U S A* (1987) **84**: 9238-9242
- Bevilacqua MP, Stengelin S, Gimbrone MA, Jr. Seed B. Endothelial leukocyte adhesion molecule 1: an inducible receptor for neutrophils related to complement regulatory proteins and lectins. *Science* (1989) **243**: 1160-1165
- Bevilacqua MP, Nelson RM, Mannori G, Cecconi O. Endothelial-leukocyte adhesion molecules in human disease. *Annu Rev Med* (1994) **45**: 361-378
- Biedermann T, Kneilling M, Mailhammer R, Maier K, Sander CA, Kollias G, Kunkel SL, Hultner L, Rocken M. Mast cells control neutrophil recruitment during T cell-mediated delayed-type hypersensitivity reactions through tumor necrosis factor and macrophage inflammatory protein 2. *J Exp Med* (2000) **192**: 1441-1452

Blankenberg S, Barbaux S, Tiret L. Adhesion molecules and atherosclerosis. *Atherosclerosis* (2003) **170**: 191-203

Blann AD, Nadar SK, Lip GY. The adhesion molecule P-selectin and cardiovascular disease. *Eur Heart J* (2003) **24**: 2166-2179

Bonecchi R, Polentarutti N, Luini W, Borsatti A, Bernasconi S, Locati M, Power C, Proudfoot A, Wells TN, Mackay C, Mantovani A, Sozzani S. Up-regulation of CCR1 and CCR3 and induction of chemotaxis to CC chemokines by IFN- $\gamma$  in human neutrophils. *J Immunol* (1999) **162**: 474-479

Boyce JA. The biology of the mast cell. *Allergy and Asthma Proc* (2004) **25**: 27-30

Burton JL, Kehrl ME, Kapil S, Horst RL. Regulation of L-selectin and CD18 on bovine neutrophils by glucocorticoids: effects of cortisol and dexamethasone. *J Leukocyte Biol* (1995) **57**: 317-325

Cacalano G, Lee J, Kikly K, Ryan AM, Pitts-Meek S, Hultgren B, Wood WI, Moor MW. Neutrophil and B cell expansion in mice that lack the murine IL-8 receptor homolog. *Science* (1994) **265**: 682-684

Carlos TM, Harlan JM. Leukocyte-endothelial adhesion molecules. *Blood* (1994) **84**: 2068-2101

Cheng SS, Lai JJ, Lukacs NW, Kunkel SL. Granulocyte-macrophage colony stimulating factor up-regulates CCR1 in human neutrophils. *J Immunol* (2001) **166**: 1178-1184

Cohan VL, Udem BJ, Fox CC, Adkinson NF Jr, Lichtenstein LM, Schleimer RP. Dexamethasone does not inhibit the release of mediators from human mast cells residing in airway, intestine or skin. *Am Rev Respir Dis* (1989) **140**: 951-954

Cronstein BN, Kimmel SC, Levin RI, Martiniuk F, Weissmann G. A mechanism for the antiinflammatory effects of corticosteroids: the glucocorticoid receptor regulates leukocyte adhesion to endothelial cells and expression of endothelial-leukocyte adhesion molecule 1 and intercellular adhesion molecule 1. *Proc Natl Acad Sci USA* (1992) **89**: 9991-9995

Davenpeck KL, Zagorski J, Schleimer RP, Bochner BS. Lipopolysaccharide-induced leukocyte rolling and adhesion in the rat mesenteric microcirculation: regulation by glucocorticoids and role of cytokines. *J Immunol* (1998) **161**: 6861-6870

Deng ZW, Denkinger DJ, Peterson KE, Deuel TF, Kawahara R. Glucocorticoids negatively regulate the transcription of KC, the mouse homolog of MGSA/GRO. *Biochem Biophys Res Commun* (1994) **203**: 1809-1814

Diab A, Abdalla H, Li HL, Shi FD, Zhu J, Hojberg B, Lindquist L, Wretling B, Bakhiet M, Link H. Neutralization of macrophage inflammatory protein2 (MIP-2) and MIP-1alpha attenuates neutrophil recruitment in the central nervous system during experimental bacterial meningitis. *Infect Immun* (1999) **67**: 2590-2601

Domachowske JB, Bonville CA, Gao JL, Murphy PM, Easton AJ, Rosenberg HF. The chemokine macrophage inflammatory protein-1 alpha and its receptor CCR1 control pulmonary inflammation and antiviral host defense in paramyxovirus infection. *J Immunol* (2000) **165**: 2677-2682

Driscoll KE, Hassenbein DC, Carter J, Poynter J, Asquith TN, Grant RA, Whitten J, Purdon MP, Takigiku R. Macrophage inflammatory protein 1 and 2: expression by rat alveolar macrophages, fibroblasts, and epithelial cells and in rat lung after mineral dust exposure. *Am J Respir Cell Mol Biol* (1993) **8**: 311-318

Ebnet K, Vestweber D. Molecular mechanism that control leukocyte extravasation: the selectins and the chemokines. *Histochem Cell Biol* (1999) **112**: 1-23

Echtenacher B, Mannel DN, Hultner L. Critical protective role of mast cells in a model of acute septic peritonitis. *Nature* (1996) **381**: 75-77

Engelhardt B, Wolburg H. Transendothelial migration of leukocytes: through the front door or around the side of the house? *Eur J Immunol* (2004) **34**: 2955-2963

Feger F, Varadaradjalou S, Gao Z, Abraham SN, Arock M. The role of mast cells in host defense and their subversion by bacterial pathogens. *Trends Immunol* (2002) **23**: 151-158

Feng L, Xia Y, Yoshimura T, Wilson CB. Modulation of neutrophil influx in glomerulonephritis in the rat with anti-macrophage inflammatory protein-2 (MIP-2) antibody. *J Clin Invest* (1995) **95**: 1009-1017

Gersch C, Dewald O, Zoerlein M, Micheal LH, Entman ML, Frangogiannis NG. Mast cell and macrophages in normal C57/BL/6 mice. *Histochem Cell Biol* (2002) **118**: 41-49

Gordon JR, Galli SJ. Mast cells as a source of both preformed and immunologically inducible TNF- $\alpha$ /cachectin. *Nature* (1990) **346**: 274-276

Gordon JR, Galli SJ. Release of both preformed and newly synthesized tumor necrosis factor  $\alpha$  (TNF- $\alpha$ )/cachectin by mouse mast cell stimulated via the Fc epsilon RI. A mechanism for the sustained action of mast cell-derived TNF- $\alpha$  during IgE-dependent biological responses. *J Exp Med* (1991) **174**: 103-107

Gupta SK, Lysko PG, Pillarisetti K, Ohlstein E, Stadel JM. Chemokine receptors in human endothelial cells. Functional expression of CXCR4 and its transcriptional regulation by inflammatory cytokines. *J Biol Chem* (1998) **273**: 4282-4287

Haddad EB, Salmon M, Sun J, Das A, Adcock I, Barnes PJ, Chung KF. Dexamethasone inhibits ozone-induced gene expression of macrophage inflammatory protein-2 in rat lung. *FEBS Lett* (1995) **363**: 285-288

Haraldsen G, Kvale D, Lien B, Farstad IN, Brandtzaeg P. Cytokine-regulated expression of E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) in human microvascular endothelial cells. *J Immunol* (1996) **156**: 2558-2565

House SD, Lipowsky HH. Leukocyte-endothelium adhesion: microhemodynamics in mesentery of the cat. *Microvasc Res* (1987) **34**: 363-379

Hsu-Lin S, Berman CL, Furie BC, August D, Furie B. A platelet membrane protein expressed during platelet activation and secretion. *J Biol Chem* (1984) **259**: 9121-9126

Huang S, Paulauskis JD, Godleski JJ, Kobzik L. Expression of macrophage inflammatory protein-2 and KC mRNA in pulmonary inflammation. *Am J Pathol* (1992) **141**: 981-988

Huber AR, Kunkel SL, Todd RF 3rd, Weiss SJ. Regulation of transendothelial neutrophil migration by endogenous interleukin-8. *Science* (1991) **254**: 99-102



Jones SA, Dewald B, Clark-Lewis I, Baggiolini M. Chemokine antagonists that discriminate between interleukine-8 receptors. Selective blockers of CXCR2. *J Biol Chem* (1997) **272**: 16166-16169

Jung U, Norman KE, Scharffetter-Kochanek K, Beaudet AL, Ley K. Transit time of leukocytes rolling through venules controls cytokine-induced inflammatory cell recruitment in vivo. *J Clin Invest* (1998) **102**: 1526-1533

Juremalm M, Nilsson G. Chemokine receptor expression by mast cells. Mast cells in Allergic Diseases. Saito H., Okayama Y. (eds). *Chem Immunol Allerg* (2005) **87**: 130-144

Kanwar S, Johnston B, Kubes P. Leukotriene C4/D4 induced P-selectin and sialyl-Lewis(x)-dependent alterations in leukocyte kinetics in vivo. *Circ Res* (1995) **77**: 879-887

Kanwar S, Steeber DA, Tedder TF, Hickey MJ, Kubes P. Overlapping roles for L-selectin and P-selectin in antigen-induced immune responses in the microvasculature. *J Immunol* (1999) **162**: 2709-2716

Kawahara RS, Deng ZW, Deuel TF. Glucocorticoids inhibit the transcriptional induction of JE, a platelet-derived growth factor-inducible gene. *J Biol Chem* (1991) **266**: 13261-13266

Kishimoto TK, Baldwin ET, Anderson DC. The role of  $\beta 2$  integrins in inflammation, *Inflammation: Basic Principles and Clinical Correlates* 3rd ed., edited by Gallin JI and Snyderman R. (1999) Chapter 37: 537-569

Klinkert WE, Kojima K, Lesslauer W, Rinner W, Lassmann H, Wekerle H. TNF- $\alpha$  receptor fusion protein prevents experimental auto-immune encephalomyelitis and demyelination in Lewis rats: an overview. *J Neuroimmunol* (1997) **72**: 163-168

Ksontini R, Mackay SLD, Moldawer LL. Revisiting the role of tumor necrosis factor  $\alpha$  and the response to surgical injury and inflammation. *Arch Surg* (1998) **133**: 558-567

Kubes P, Granger DN. Leukocyte-endothelial cell interactions evoked by mast cells. *Cardiovascular Res* (1996) **32**: 699-708

Kunkel EJ, Dunne JL, Ley K. Leukocyte arrest during cytokine-dependent inflammation in vivo. *J Immunol* (2000) **164**: 3301-3308

- Lasky LA. Selectin-carbohydrate interactions and the initiation of the inflammatory response. *Annu Rev Biochem* (1995) **64**: 113-139
- Leng W, Kuo CG, Qureshi R, Jakschik BA. Role of leukotrienes in vascular changes in the rat mesentery and skin in anaphylaxis *J Immunol* (1988) **140**: 2361-2368
- Ley K, Allietta M, Bullard DC, Morgan S. Importance of E-selectin for firm leukocyte adhesion in vivo. *Cir Res* (1998) **83**: 287-294
- Ley K, Bullard DC, Arbones ML, Bosse R, Vestweber D, Tedder TF, Beaudet AL. Sequential contribution of L- and P-selectin to leukocyte rolling in vivo. *J Exp Med* (1995) **181**: 669-675
- Lindbom L, Xie X, Raud J, Hedqvist P. Chemoattractant-induced firm adhesion of leukocytes to vascular endothelium in vivo is critical dependent on initial leukocyte rolling. *Acta Physiol Scand* (1992) **146**: 415-421
- Lippert U, Artuc M, Grutzkau A, Moller A, Kenderessy-Szabo A, Schadendorf D, Norgauer J, Hartmann K, Schweitzer-Stenner R, Zuberbier T, Henz BM, Kruger-Krasagakes S. Expression and functional activity of the IL-8 receptor type CXCR1 and CXCR2 on human mast cells. *J Immunol* (1998) **161**: 2600-2608
- Mansson P, Zhang XW, Jeppsson B, Johnell O, Thorlacius H. Critical role of P-selectin-dependent rolling in tumor necrosis factor- $\alpha$  induced leukocyte adhesion and extravascular recruitment in vivo. *Naunyn Schmiedebergs Arch Pharmacol* (2000) **362**: 190-196
- Malaviya R, Ikeda T, Ross E, Abraham SN. Mast cell modulation of neutrophil influx and bacterial clearance at sites of infection through TNF- $\alpha$ . *Nature* (1996) **381**: 77-80
- Matsushima H, Yamada N, Matsue H, Shimada S. TLR3-, TLR7-, and TLR9-mediated production of proinflammatory cytokines and chemokines from murine connective tissue type skin-derived mast cells but not from bone marrow-derived mast cells. *J Immunol* (2004) **173**: 531-541
- Matsukawa A, Hogaboam CM, Lukacs NW, Lincoln PM, Strieter RM, Kunkel SL. Endogenous monocyte chemoattractant protein-1 (MCP-1) protects mice in a model of acute septic peritonitis: cross-talk between MCP-1 and leukotriene B4. *J Immunol* (1999) **163**: 6148-6154

Mayadas TN, Cullere X. Neutrophil  $\beta 2$  integrins: moderators of life or death decisions. *Trends Immunol* (2005) **26**: 388-395

McColl SR, Hachicha M, Levasseur S, Neote K, Schall TJ. Uncoupling of early signal transduction events from effector function in human peripheral blood neutrophils in response to recombinant macrophage inflammatory protein-1 alpha and -1 beta. *J Immunol* (1993) **150**: 4550-4560

McColl SR, Clark-Lewis I. Inhibition of murine neutrophil recruitment in vivo by CXC chemokine receptor antagonists. *J Immunol* (1999) **163**: 2829-2835

McEver RP. Selectins. *Curr Opin Immunol* (1994) **6**: 75-84

McEver RP, Beckstead JH, Moor KL, Marshall-Carlson L, Bainton DF. GMP-140, a platelet alpha-granule membrane protein, is also synthesized by vascular endothelial cells and is localized in Weibel-Palade bodies. *J Clin Invest* (1989) **84**: 92-99

Mercer-Jones MA, Shrotri MS, Heinzelmann M, Peyton JC, Cheadle WG. Regulation of early peritoneal neutrophil migration by macrophage inflammatory protein-2 and mast cells in experimental peritonitis. *J Leukoc Biol* (1999) **65**: 249-255

Milstone DS, Fukumura D, Padgett RC, O'Donnell PE, Davis VM, Benavidez OJ, Monsky WL, Melder RJ, Jain RK, Gimbrone MA Jr. Mice lacking E-selectin show normal numbers of rolling leukocytes but reduced leukocyte stable arrest on cytokine-activated microvascular endothelium. *Microcirculation* (1998) **5**: 153-171

Moore KL, Patel KD, Bruehl RE, Li F, Johnson DA, Lichenstein HS, Cummings RD, Bainton DF, McEver RP. P-selectin glycoprotein ligand-1 mediates rolling of human neutrophils on P-selectin. *J Cell Biol* (1995) **128**: 661-671

Moore TA, Newstead MW, Strieter RM, Mehrad B, Beaman BL, Standiford TJ. Bacterial clearance and survival are dependent on CXC chemokine receptor-2 ligands in a murine model of pulmonary *Nocardia asteroides* infection. *J Immunol* (2000) **164**: 908-915

Mori N, Horie Y, Gerritsen ME, Anderson DC, Granger DN. Anti-inflammatory drugs and endothelial cell adhesion molecule expression in murine vascular beds. *Gut* (1999) **44**: 186-195

Muller WA. Leukocyte-endothelial cell adhesion molecules in transendothelial migration, *Inflammation: Basic Principles and Clinical Correlates* 3rd ed., edited by Gallin JI and Snyderman R. (1999) Chapter 39: 585-592

Muller WA. Leukocyte-endothelial cell interactions in leukocyte transmigration and the inflammatory response. *Trends Immunol* (2003) **24**: 327-334

Murdoch C, Monk PN, Finn A. CXC chemokine receptor expression on human endothelial cells. *Cytokine* (1999) **11**: 704-712

Nilsson G, Mikovits JA, Metcalfe DD, Taub DD. Mast cell migratory response to interleukin-8 is mediated through interaction with chemokine receptor CXCR2/Interleukin-8RB. *Blood* (1999) **93**: 2791-2797

O'Leary EC and Zuckerman SH. Glucocorticoid-mediated inhibition of neutrophil emigration in an endotoxin-induced rat pulmonary inflammation model occurs without an effect on airways MIP-2 levels. *Am J Respir Cell Mol Biol* (1997) **16**: 267-274

Paton WD. Compound 48/80: A potent histamine liberator. *Br J Pharmacol* (1951) **6**: 499-508

Petzelbauer P, Watson CA, Pfau SE, Pober JS. IL-8 and angiogenesis: evidence that human endothelial cells lack receptors and do not respond to IL-8 in vitro. *Cytokine* (1995) **7**: 267-272

Pober JS, Gimbrone MA Jr, Lapierre LA, Mendrick DL, Fiers W, Rothlein R, Springer TA. Overlapping patterns of activation of human endothelial cells by interleukin 1, tumor necrosis factor and immune interferon. *J Immunol* (1986) **137**: 1893-1896

Ramos BF, Zhang Y, Qureshi R, Jakschik BA. Mast cells are critical for production of leukotrienes responsible for neutrophil recruitment in immune complex-induced peritonitis in mice. *J Immunol* (1991) **147**: 1636-1641

Robinson SD, Frenette PS, Rayburn H, Cumiskey M, Ullman-Cullere M, Wagner DD, Hynes RO. Multiple, targeted deficiencies in selectins reveal a predominant role for P-selectin in leukocyte recruitment. *Proc Natl Acad Sci U S A* (1999) **96**: 11452-11457

Robinson LA, Steeber DA, Tedder TF. The selectin in inflammation, *Inflammation: Basic Principles and Clinical Correlates* 3rd ed., edited by Gallin JI and Snydermn R. (1999) Chapter 38: 571-605

Rollins BJ, Chemokines. *Blood* (1997) **90**: 909-928

Schleimer RP. An overview of glucocorticoid anti-inflammatory actions. *Eur J Clin Pharmacol* (1993) **45**: (suppl. 1) S 3-7

Schmal H, Shanly TP, Jones ML, Friedl HP, Ward PA. Role for macrophage inflammatory protein-2 in lipopolysaccharide-induced lung injury in rats. *J Immunol* (1996) **156**: 1963-1972

Schonbeck U, Brandt E, Petersen F, Flad HD, Loppnow H. IL-8 specifically binds to endothelial but not to smooth muscle cells. *J Immunol* (1995) **154**: 2375-2383

Schramm R, Schaefer T, Menger MD, Thorlacius H. Acute mast cell-dependent neutrophil recruitment in the skin is mediated by KC and LFA-1: inhibitory mechanism of dexamethasone. *J Leukoc Biol* (2002) **72**: 1122-1132

Schramm R, Thorlacius H. Neutrophil recruitment in mast cell-dependent inflammation: inhibitory mechanisms of glucocorticoids. *Inflamm Res* (2004) **53**: 644-652

Shanley TP, Schmal H, Friedl HP, Jones ML, Ward PD. Role of macrophage inflammatory protein-1alpha (MIP-1 $\alpha$ ) in acute lung injury in rats. *J Immunol* (1995) **154**: 4793-4802

Shimizu H, Nagakui Y, Tsuchiya K, Horii Y. Demonstration of chymotryptic and tryptic activities in mast cells of rodents: comparison of 17 species of the family muridae. *J Comp Path* (2001) **125**: 76-79

Simon SI, Hu Y, Vestweber D, Smith CW. Neutrophil tethering on E-selectin activates beta 2 integrin binding to ICAM-1 through a mitogen-activated protein kinase signal transduction pathway. *J Immunol* (2000) **164**: 4348-4358

Smart SJ, Casale TB. TNF- $\alpha$ -induced transendothelial neutrophil migration is IL-8 dependent. *Am J Physiol* (1994) **266**: L238-245

Spada CS, Krauss AH, Nieves AL, Woodward DF. Effects of leukotrienes B4 (LTB4) and D4 (LTD4) on motility of isolated normodense human eosinophils and neutrophils. *Adv Exp Med Biol* (1997) **400B**: 699-706

Taylor A, Tomlinson A, Salas A, Panes J, Granger DN, Flower RJ, Perretti M. Dexamethasone inhibition of leukocyte adhesion to rat mesenteric postcapillary venules: role of intercellular adhesion molecule 1 and KC. *Gut* (1999) **45**: 705-712

Takeda K, Kaisho T, Akira S. Toll-like receptors. *Annu Rev Immunol* (2003) **21**: 335-376

Tang T, Frenette PS, Hynes RO, Wagner DD, Mayadas TN. Cytokine-induced meningitis is dramatically attenuated in mice deficient in endothelial selectins. *J Clin Invest* (1996) **97**: 2485-2490

Tekamp-Olson P, Gallegos C, Bauer D, McClain J, Sherry B, Fabre M, van Deventer S, Cerami A. Cloning and characterization of cDNAs for murine macrophage inflammatory protein 2 and its human homologues. *J Exp Med* (1990) **172**: 911-919

Tessier PA, Naccache PH, Clark-Lewis I, Gladue RP, Neote KS, McColl SR. Chemokine networks in vivo: involvement of C-X-C and C-C chemokines in neutrophil extravasation in vivo in response to TNF- $\alpha$ . *J Immunol* (1997) **159**: 3595-3602

Tessier PA, Naccache PH, Diener KR, Gladue RP, Neote KS, Clark-Lewis I, McColl SR. Induction of acute inflammation in vivo by staphylococcal superantigens. II. Critical role for chemokines, ICAM-1, and TNF- $\alpha$ . *J Immunol* (1998) **161**: 1204-1211

Theoharides TC and Cochrane DE. Critical role of mast cells in inflammatory disease and the effect of acute stress. *J Neuroimmunol* (2003) **146**: 1-12

Thorlacius H. Mediator synergism in mast cell-dependent inflammation, Role of histamine for leukocyte recruitment. *Thesis* (1996) P.13

Thorlacius H, Raud J, Rosengren-Breezley S, Forrest MJ, Hedqvist P, Lindbom L. Mast cell activation induces P-selectin-dependent leukocyte rolling and adhesion in postcapillary venules in vivo. *Biochem Biophys Res Commun* (1994) **203**: 1043-1049

Thorlacius H, Raud J, Xie X, Hedqvist P, Lindbom L. Microvascular mechanism of histamine-induced potentiation of leukocyte adhesion evoked by chemoattractants. *Br. J Pharmacol* (1995) **116**: 3175-3180

Tuckermann JP, Kleiman A, McPherson KG, Reichardt HM. Molecular mechanism of glucocorticoids in the control of inflammation and lymphocyte apoptosis. *Crit Rev Clin Lab Sci* (2005) **42**: 71-104

van Deventer SJ. Tumor necrosis factor and Crohn's disease. *Gut* (1997) **40**: 443-448

van Mourik JA, Romani de WT, Voorberg J. Biogenesis and exocytosis of Weibel-Palade bodies. *Histochem Cell Biol* (2002) **117**: 113-122

Vestweber D and Blanks JE. Mechanisms that regulate the function of the selectins and their ligands. *Physiol Res* (1999) **79**: 181-213

Wan MX, Riaz AA, Schramm R, Wang Y, Vestweber D, Menger MD, Thorlacius H. Leukocyte rolling is exclusively mediated by P-selectin colonic venules. *Br J Pharmacol* (2002) **135**: 1749-1756

Weller A, Isenmann S, Vestweber D. Cloning of the mouse endothelial selectins. Expression of both E- and P-selectin is inducible by tumor necrosis factor alpha. *J Biol Chem* (1992) **267**: 15176-15183

Williams RL, Risau W, Zerwes HG, Drexler H, Aguzzi A, Wagner EF. Endothelioma cells expressing the polyoma middle T oncogene induce hemangiomas by host cell recruitment. *Cell* (1989) **57**: 1053-1063

Yamaki K, Lindbom L, Thorlacius H, Hedqvist P, Raud J. An approach for studies of mediator-induced leukocyte rolling in the undisturbed microcirculation of the rat mesentery. *Br J Pharmacol* (1998a) **123**: 381-389

Yamaki K, Thorlacius H, Xie X, Lindbom L, Hedqvist P, Raud J. Characteristics of histamine-induced leukocyte rolling in the undisturbed microcirculation of the rat mesentery. *Br J Pharmacol* (1998b) **123**: 390-399

Yi ES, Remick DC, Lim Y, Tang W, Nadzienko CE, Bedoya A, Yin S, Ulich TR. The intratracheal administration of endotoxin: X. Dexamethasone downregulates neutrophil emigration and cytokine expression in vivo. *Inflammation* (1996) **20**: 165-175

Zhang XW, Thorlacius H. Dexamethasone inhibits arteriolar leukocyte rolling and adhesion induced by tumor necrosis factor-alpha in vivo. *Inflamm Res.* (2000) **49**:95-97

Zhang Y, Ramos BF, Jakschik BA. Neutrophil recruitment by tumor necrosis factor from mast cells in immune complex peritonitis. *Science* (1992) **258**: 1957-1959

Zhelev DV, Alteraifi A. Signaling in the motility responses of the human neutrophil. *Ann Biomed Eng* (2002) **30**: 356-370

Zlotnik A, Morales J, Hedrick JA. Recent advances in chemokines and chemokine receptors. *Crit Rev Immunol* (1999) **19**: 1-47